The effects of burying beetle social behaviours on interspecific interactions

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Chapter 1

Introduction

Overview on insect microbiomes

The microbiome of animals consists of the community of microbes that colonize host organisms, including the complete set of commensal, symbiotic and harmful species (1–3). This ecological community has long been known to affect host biology, and their diverse roles have been further clarified in recent years following numerous studies of animal:microbiota interactions in diverse systems (4–6). The models used to study host-microbiota interactions have covered a broad range of animal taxa, such as nematodes, bobtail squid, insects, zebra fish, mouse and human beings, among others (7). Using these models, scientists are drawing on interdisciplinary approaches and techniques across ecology, bioinformatics and biomedical science to gain deeper knowledge about the biological significance of host-microbe symbioses.

Insects are the largest animal group on earth, and feature varied symbiotic associations with microbial species (8). Insect microbiota colonize the external surface and also reside internally in specialized compartments of insects, such as the external carapace and the bacteriocites inside the body (9–11). The intestinal gut tract provides an ecological niche for microbes. Bacterial densities within insect guts differ broadly across host species ranging from 10^5-10^9 cells per gut, with species like the fruit fly *Drosophila* containing a bacterial density of about 10^5 bacteria (9, 12). The guts of some sap-feeding aphids may even be sterile (13). In contrast to the rich microbial diversity in vertebrate guts which can contain > 500 taxa, most insects harbour relatively limited bacterial diversity (14, 15). For example, there are only 1~30 taxa of microbes found in the gut of *Drosophila* (16). By contrast, some eusocial insects i.e. honey bees and wood-feeding termites, contain a more diverse community of bacteria within their gut microbiota, with more than 300 and 367 identified phylogenetic clusters, respectively, in worker honey bees and lower termites (7, 12, 17, 18). It has been suggested that eusociality may enhance opportunities for microbiota transmission between colony members, and thus promote a diverse gut microbiome. In spite of their limited diversity, insect microbiota

can have dramatic effects on their hosts, in terms of nutrition, development, immune response, morphogenesis and behavior, among others (19–23). And in return, these effects might also influence the colonization and composition of insect microbiota (12, 24–26).

For example, the honey bee gut microbiota can promote host physiology by increasing host weight, hormonal signaling and sucrose sensitivity (27). The fungus Beauveria, which resides in the breeding environment of dung beetles *Euoniticellus intermedius*, stimulates the immune response by triggering Toll signaling of beetles to fight against microbial infections (28). Other microbial symbionts can modify animal behaviour. For example, the bacteria *Proteus mirabilis* can produce volatiles to attract blow flies, who can transport this bacteria to new food resources; in return, blow flies use the chemical cues from bacteria *P. mirabilis* to locate food resources (29). In addition, some microbes residing in termites guts can help the host to digest lignocellucose and thus play essential roles in nutrient metabolism (30). Other bacteria from *Lactobacillales* and *Acetobacteraceae* activate the TOR pathway in *Drosophila*, which regulate the hormonal signals involved in molting (31). In addition, insect microbiota have been also reported to influence macro-evolutionary processes of hosts by promoting the divergence of host lineages and speciation. For example, symbiont-mediated changes in host behavior, e.g. mate choice, may lead to host reproductive isolation (32). In addition, the long-term colonization of microbiota within hosts can result in host-microbiota co-speciation over millions of years (seen as congruent phylogenies), which has been reported in many insect species, such as aphids, bees and mealybugs (33–35). For instance, Moran (36) has described the co-speciation between sap-feeding aphids and their obligate nutritional mutualist *Buchnera*, and suggests that their symbiotic association began more than 50 million years ago (37). Despite these wide-ranging influences, the effects of insect microbiota may be broader still, and more studies are needed to explore the diverse roles of the microbiota on hosts with different life-histories, which will enable us to better understand general and specific features of insect-microbiota ecology.

In this thesis, I will shed light on the ecological interactions between the burying beetle, *Nicrophorus vespilloides*, and its gut microbiota. I will investigate the potential mechanisms underlying the transmission and colonization of gut microbiota of this species. In addition, I will examine some

of the effects conferred by the beetle's microbiota on its ecology. My research highlights the association between host behaviour and gut microbiota ecology.

Transmission and colonization of insect gut microbiota

Insects can acquire their microbiota through either vertical or horizontal transmission. Vertical transmission refers to cases where parents transmit their gut microbiota to their offspring directly via the egg or egg coat, while insects that rely on horizontal transmission acquire their microbiota from the environment, including from other individuals (38, 39). However, some insects fall in the middle of these strict extremes. For example, honeybee workers *Apis mellifera* acquire and establish their gut communities via different routes including mouth-mouth or anal-mouth transmission between nest members (trophallaxis), and fecal consumption from the environment (coprophagy) (40, 41). In addition, the modes of microbiota transmission can vary significantly even among closely related insects. For instance, many stinkbugs initiate the vertical transmission of their core microbiota by excreting anal secretions to either the egg surface or offspring larvae (42). In contrast, the stinkbug *Riptortus clavatus* acquire their beneficial symbiont *Burkholderia* obligately from the environment (43). A similar mode of transmission might be used by the stinkbug *Megacopta punctatissima*. If the new born nymphs receive no parental provisioning for the gut symbionts, they will show more wandering behaviour which potentially facilitates the acquisition of their gut symbionts (44).

During insect gut microbiota establishment, different insect life styles, e.g. solitary or eusocial, can be an important factor facilitating the transmission and thus the composition of gut microbiota. For example, *Drosophila melanogaster* replenish their obligate symbionts via food consumption (45); bumble bees (*Bombus terrestris*) obtain their gut microbiota via contact with nest mates via trophallaxis, and reduced association with nest mates will result in an alteration of their gut microbiota (46). Although the association between host behaviour and microbiota transmission varies through different systems, host behaviour and microbiota transmission could co-evolve for the benefits of both sides of host-microbiomes (47, 48). In this thesis, I will investigate the transmission mechanisms of *N. vespilloides* gut microbiota during development, and demonstrate how this is associated with host social behaviour and developmental transitions.

Factors that influence the insect gut microbiota

Multiple factors influence the establishment and subsequent maintenance of the insect gut community. Any change of these factors, such as diet, habitat, social interactions and microbial or parasitic infections to insects can alter colonization dynamics and shift the gut microbiota in density and/or diversity (49–51). Another important factor that influences the microbiome is development (20). Holometabolous insects go through several molting stages during their development from eggs to adults that are usually accompanied with significant changes to gut structure and condition (52, 53). Insects with a complete metamorphosis undergo 4 stages of development in general, including egg, larva, pupa and adult. For some insect species, e.g. moth, butterfly and *Coleoptera* beetles, they usually undergo a pre-pupa and an adult-eclosion stage before and after the pupa imago (54–56). Hemimetabolous insects with incomplete metamorphosis go through a transition simply from egg to nymph then to adult, and there is no pupal stage during this metamorphosis (52, 53). Despite the differences in metamorphic types, both hemimetabolous and holometabolous processes require that insects shed their exoskeleton during the molting stage and this includes the lining of the fore and hind gut epithelium (57, 58). Thus, over the course of development, the entire intestinal tract is shed during the process (57, 59). The reformation of the adult intestinal tract prior to adult eclosion results in a series of changes in gut physiology and contents, such as the size of epithelium cells and the activity of metabolic enzymes (12, 60, 61). These physiological changes potentially result in alterations to the insect microbiota in both abundance and structure (12, 62). For example, in the newly emerged adult mosquito Culicidae, a nearly complete clearance of bacteria is found in the mid-gut (63). Similarly, a molting mediated reduction of gut symbionts has been also found in bean bugs *Riptortus pedestris* (64). By contrast, gut bacteria persist through stages of housefly development (65). Hence, while developmental shifts can alter gut microbiota dynamics, the affects are neither universal nor predictable. One of the aims of this thesis is to quantify the dynamics of gut bacteria across *N. vespilloides* developmental stages via profiling of cultured microbes, and to illuminate how microbiome dynamics are influenced by host metamorphosis and behaviour.

In addition to host and environmental factors, microbial interactions within the gut could also mediate the variation in composition and abundance

of gut microbiota, and thus play an important role in their maintenance and stability. Coyte et al (2015) suggest that bacterial competition within the gut could increase gut microbiota stability as compared to bacterial cooperative interactions. Their model suggests that hosts could interfere with the interactions taking place between members of their microbiomes and thereby manipulate the microbial communities to their benefit (66). Another example from Bombus bees found that the richness of non-core microbiota negatively associates with microbiota abundance in the gut, which also suggests the structure of gut microbiota might be facilitated by host-influenced microbial interactions within the gut (67). Despite the understanding that both insect development and microbial interactions could influence the composition and the maintenance of the gut microbiome, few studies have shown the details about how the colonization of gut microbes changes throughout the entirety of insect development, which is important if we are to comprehend how the microbiota persist.

Colonization resistance of insect gut microbiota

While the microbiota can be helpful to insects, insects will still encounter harmful microbes that can be pathogenic. These harmful species can infect insects and possibly colonize their guts. Such colonization may result in bacterial competition within the gut between resident species and potential pathogens and may cause community-level changes to the gut microbiota (67). Insects have evolved diverse strategies to overcome threats from such harmful microbes. As one of these strategies, colonization resistance has been observed in many animal gut communities. Colonization resistance is a mechanism whereby resident microbiota resist against subsequent microbial colonization, including pathogens, following exposure. The endogenous microbiota of insects such as sand flies, silk worms and desert locusts, help their hosts to resist against pathogen colonization of the gut (68–70), and the ability to resist challenge may scale with the diversity of the gut microbiome (71). For example locusts, *Schistocerca gregaria*, with more diverse gut symbionts are better able to reduce the density of the pathogen *Serratia marcescens* during experimental colonization of the gut (70). Colonization resistance is known to protect hosts from microbial infection and play an important role in the stability of the host gut microbiota and thus host health (72, 73).

The mechanisms of colonization resistance vary in different host systems

and can be driven by either direct or indirect factors. In some cases of colonization resistance, the resident microbiota can directly compete with foreign microbes for the gut niche or inhibit them directly by producing bacteriocins or antibiotics (74). For other cases, resident species mediate host immunity that initiate a type of indirect resistance against the colonizing microbes. These have been found in both mammals and insects. For instance, the native gut microbiota in honey bees have been reported to induce the expression of antimicrobial peptides (AMPs) in host gut tissue (75).

Colonization resistance can also be influenced by two ecological factors: priority effects and specificity effects. Priority effects in gut communities suggests that the first bacterial colonizer within the gut persists challenge by virtue of being there first, which allows it to shape the inner intestinal environment and influence the establishment of later communities (76). Conversely, specific effects indicates that some gut bacterial species are competitively superior in a given host no matter what the colonization sequence is (77). In Chapter 4 of my thesis, I will examine if colonization resistance of bacteria occurs within the *N. vespilloides* gut, and I test which of these two ecological factors most contribute to *N. vespilloides* gut microbiota transmission and colonization, and further how these affect host fitness.

Nicrophorus vespilloides

The burying beetle *Nicrophorus vespilloides* (Coleoptera, Silphidae) is a holometabolous insect, which undergoes a complete metamorphosis (Figure 1). After hatching, larvae transition through several larval moults, which is followed by pupation and then eclosion as an adult. Beetles are reared on decomposing carcasses of small mammals or birds that are detected by breeding adults using volatiles emitted from the carcass (78, 79). These beetles evolved sophisticated parental care behaviors during breeding which is usually divided into two phases: pre-hatch care and post-hatch care (80). Pre-hatch care starts before oviposition, and consists of a series of manipulations that prepare the carcass for burial and the arrival of larvae. Adult beetles first bury the carcass into a shallow grave, then strip off the fur and roll the carcass into a ball, after which they open a hole on the carcass abdomen for access to the offspring (81, 82). At the same time, parental beetles cover the carcass with oral and anal secretions, containing a lysozyme-like compound as well as other compounds that are used to defend against bacterial and fungal competitors

(81, 83). Beetle eggs are laid nearby the carcass, and newly hatched larvae migrate to the prepared carcass for development. After larvae arrive to the carcass, parental beetles continue to provide post-hatch care to their offspring by defending them from other insect predators and feeding the larvae though direct regurgitation (Figure 2A) (79, 80, 84). In the lab, approximately 7 days post-hatching, individual larvae disperse from the carcass and then construct a chamber for pupation. The whole period of pupation usually takes around 2-3 weeks (Figure 2B, C), after which the newly eclosed adults emerge from soil. The entire duration of larval development lasts around one month, but its length varies through seasons in nature (85, 86).

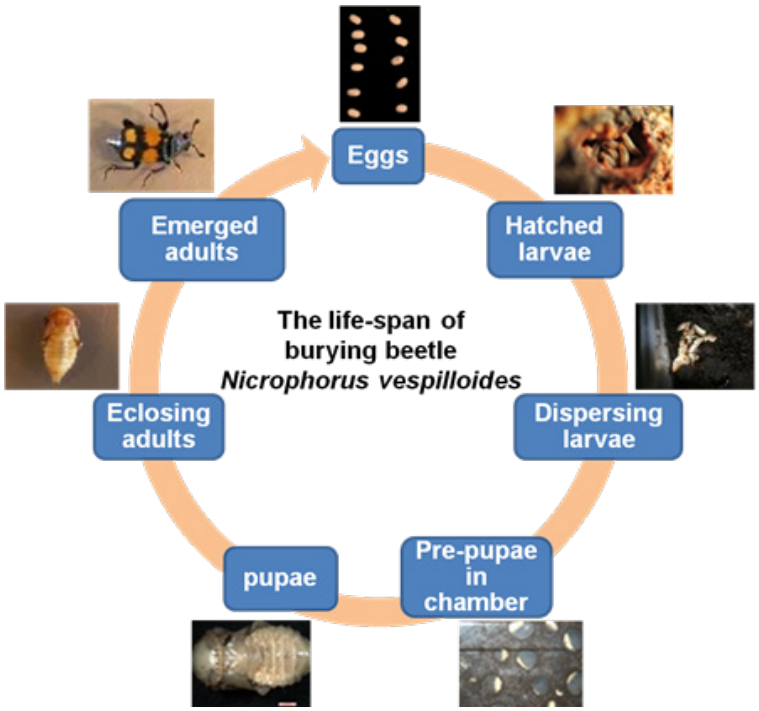

Figure 1. The life-span of burying beetle *N.vespilloides*.

Interspecific interactions across *N. vespilloides* development

Burying beetles *N. vespilloides* associate with diverse species during their life span, including microbes, nematodes and phoretic mites. Beetles feed and reproduce on carrion, and thus are exposed to and compete with diverse communities of bacterial decomposers (79, 87–89). Previous work has shown

that beetle larvae are harmed during these interactions and also that parental pre-hatch care and up-regulated immunity can partly reduce the threats from potentially pathogenic microbes (83, 88). In this thesis I examine the further protective role potentially provided by the beetles' gut microbiota. In addition to microbial interactions, Sloan Wilson examined the associations between *N. vespilloides* and phoretic mites, and shed light on the ecological effects of mites on beetle biology (90). Kilner further examined the diverse species of *N. vespilloides* associated mites and extended knowledge about their influence on the parental care of *N. vespilloides* (91–93). Although nematodes have been reported in association with *N. vespilloides* (94), we still lack an understanding of their effects on beetles social ecology.

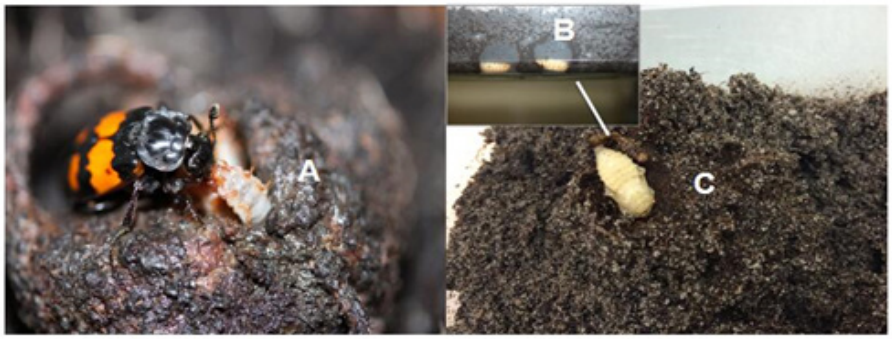

Figure 2. Morphology of the burying beetle *N. vespilloides* in larval and pupa stages of development. (A) parental regurgitation to larvae. (© Per T Smiseth); (B) larval beetles during pupation in their chambers; (C) beetle pupa inside the chamber.

Bacteria: Small vertebrate carcasses utilized by *Nicrophorus* for breeding become populated by saprophytic and pathogenic bacteria and fungi, which can harm *Nicrophorus* beetles and compromise their development and health (81, 88, 95). *Nicrophorus* eggs are initially laid nearby the carcass where microbial densities can be quite high due to nutrient pools that accumulate from the carcass (79). Studies have shown that competition with microbes from highly decomposing carcasses (aged carcasses) reduce both brood size and larval mass of *N. vespilloides* (88). In response, *N. vespilloides* reduces and avoids these threats via diverse antimicrobial strategies. For example, the direct fight against carcass derived microbiota via personal or social immunity occurs during offspring development (83, 96). Parental beetles prepare the carcass and continuously apply exudates, including e.g. lysozyme

onto the carcass surface (83, 88). Meanwhile, offspring larvae contribute to the social immunity by secreting their own anal secretions onto the carcass (83). At present there is limited understanding of how parental manipulations of the carcass influence the composition of the carcass bacterial community (84, 97). In addition, we lack an understanding about the potential interactions between the carcass microbiota and the endogenous gut symbionts of the beetles. The endogenous gut communities of *N. vespilloides* predominantly consist of *Proteobacteria*, *Firmicutes*, *Bacteriodetes* and *Actinobacteria* (89). Recent research from Kilner's group, consistent with the work presented in Chapter 3 of my thesis, shows that the most abundant bacterial taxonomic units from both adult and larval beetles are related to *Providencia spp*., *Morganella morganii*, *Proteus spp*., *Vagococcus spp*., *Clostridium spp*. and *Neisseria spp* (97). These results, based on sequencing, are consistent with my data using culture-based approaches. In my thesis, I look at the dynamics of *N. vespilloides* gut microbiota colonization and clarify when and how parental beetles transmit their bacteria to larval offspring. The potential benefits of endogenous bacteria in *Nicrophorus* will be also examined.

Mites and Nematodes: Phoretic mites and nematodes can be found in association with many insects and in many contexts (98, 99). Phoretic species hitch a ride on insect hosts for transmission and dispersal to new resources (100). *N. vespilloides* associates with many different mite species in nature (90). Notably, these mites species vary from harmful to neutral or even beneficial to *N. vespilloides*, depending on the density and species of mites (90, 92). A recent study indicates that the association with *Poecilochirus carabi* mites interferes with both parental behavior and larval fitness, and high mite densities changes the trade-off between the total brood size and larval mass of the developing larvae (91–93, 101).

In addition to mites, Ritcher in 1993 first reported the association between *N. vespilloides* and the apparently phoretic nematode species *Rhabditis stammeri* (94). Although nematodes have been found to be commonly associated with *N. vespilloides* in the field, it is not known how widespread *R. stammeri* is or if other nematode species may associate with *N. vespilloides*. Furthermore, the potential fitness consequences of the association between beetles and nematodes have never been characterized. Scarab beetles show carriage of different nematode species, including either *necromenic* species or entomopathogenic species (102). *Necromenic* nematodes are species that

consume the microbes growing within the host cadaver. While in some cases they harmoniously coexist with the host until the host dies, others secrete bacteria to speed up host death, which suggests a transition from the neutral towards the entomopathogenic (or parasitic) species in nematodes (103, 104).

Aim and outline of this thesis

The aim of the thesis is to experimentally investigate the origin and consequences of different interspecific interactions within burying beetle *N. vespilloides* social ecology. Parental care is an important factor in this species and thus may impact these interspecific interactions. My thesis will especially help us to better understand how parental behaviour impacts gut microbiota transmission and colonization.

According to the previous results we obtained, the hypotheses of this research are that 1) gut symbionts of *N. vespilloides* will benefit host fitness; and 2) symbiont transmission to offspring is facilitated by parental care. To target our research goals and examine our hypotheses, we thus put forward the following questions:

1. What are the dynamics of transmission of *N. vespilloides* symbionts to larvae during development?
2. What are the fitness effects to larvae of retaining the "endogenous" gut microbiota?
3. How does the "endogenous " microbiota persist in the *N. vespilloides* gut?
4. How do other interspecific associations affect *N. vespilloides* ecology?

In **CHAPTER 2** I evaluate the challenge of the carcass associated soil environment to *N. vespilloides* egg survival and examine potential antimicrobial strategies of *N. vespilloides* eggs. I first examine egg survival with different levels of microbial exposure. I next test the immune response of *Nicrophorus* eggs and newly hatched larvae. Further, I investigate whether the immunologically active physical barrier called the Serosa exists in *N. vespilloides* eggs. In light of these results, I discuss evolutionary consequences of antimicrobial activities of *N. vespilloides* in their early life stages.

In **CHAPTER 3** I clarify the transmission mechanisms of *N. vespilloides*

gut microbiota and demonstrate the role of parental care in this transmission. I first illuminate changes in the density of gut microbiota during the development of *N. vespilloides*, and then manipulate *N. vespilloides* parental care to offspring larvae, and monitor the composition of larval gut symbionts through development. Further, I conclude that *N. vespilloides* undergoes a significant aposymbiotic stage during pupation, after which they are recolonized at eclosion with bacteria similar to those found on the molted larval cuticle and on the wall of the pupal chamber. In addition, I clarify the importance of pre-hatch care on the transmission and colonization of *N. vespilloides* gut microbiota.

CHAPTER 4 focuses on questions about the fitness effects of the indigenous bacteria to parental and larval *N. vespilloides* and the mechanisms underlying the persistence of *N. vespilloides* gut bacteria. I first assess the general effects of endogenous microbiota on larval fitness. Next, I take a closer look at the impact of different bacterial symbionts on larval survival through time. Last, I conduct bacterial competition assays within the larval intestinal environment and estimate the colonization resistance of gut symbionts. I carry out these tests on two "endogenous" species and two environmental bacterial species, including pathogens. First, I show that beetles colonized by their endogenous microbiota produce heavier broods than those colonized with carcass bacteria. Next I show that the endogenous bacterial species are better colonizers within the beetle gut. Finally, I find that the endogenous species outcompete the carcass bacterial species in the larval gut and thus provide beetles with colonization resistance against pathogens. A priority effect is suggested within the bacterial competition in the beetle gut.

The last experimental part of this thesis (**CHAPTER 5**) examines the influence of nematodes on *Nicrophorus* fitness. I first characterize the efficacy of nematode transmission across partners and generations during *Nicrophorus* breeding. I next show that this interspecific interaction significantly harmful to *Nicrophorus* parental fitness. Finally, I provide the first report a new species of nematode symbiont in *N. vespilloides*.

Finally, in **CHAPTER 6** of this thesis, all the findings are summarized. I focus on the interactions between gut symbiont ecology and burying beetle *N. vespilloides* parental behaviour. I also highlight the ecological significance of bacterial competition derived colonization resistance against pathogens in the

beetle gut. I further discuss the potential to better mimic *Nicrophorus* natural conditions for future research, and elucidate potential host-microbiota co-evolved factors that influence *Nicrophorus* gut ecology. Last, I discuss the causes of harmful nematode species to *N. vespilloides* and suggest a further investigation of nematode infections on developing larvae.

My work will significantly advance our understanding of the evolution of mutualistic gut flora in insects, as well as the relevance of social behaviour for the transmission of animal bacterial symbionts. In addition, my results will highlight the need to integrate symbiont microbiology and behavioral ecology to better understand insect ecology and evolution. Detailed analysis of the interplay between *N. vespilloides* and their bacterial symbionts may identify novel mechanisms of colonization resistance, and establish the framework for similar studies in other animal:symbiont associations.

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Chapter 2

Egg survival is reduced by grave-soil microbes in the carrion beetle, *Nicrophorus vespilloides*

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Abstract

Nicrophorus vespilloides eggs are deposited into the soil in close proximity to the decomposing vertebrate carcasses that these insects use as an obligate resource to rear their offspring. Eggs in this environment potentially face significant risks from the bacteria that proliferate in the grave-soil environment following nutrient influx from the decomposing carcass. Our aims in this paper are twofold: first, to examine the fitness effects of grave-soil bacteria to eggs, and second, to quantify egg immunocompetence as a defence against these bacteria.

Our results provide strong evidence that grave-soil microbes significantly reduce the survival of *Nicrophorus* eggs. Females provided with microbe rich carcasses to rear broods laid fewer eggs that were less likely to hatch than females given uncontaminated carcasses. Furthermore, we show that egg hatch success is significantly reduced by bacterial exposure. Using a split-brood design, which controlled for intrinsic differences in eggs produced by different females, we found that eggs washed free of surface-associated bacteria show increased survival compared to unwashed eggs. By contrast, eggs exposed to the entomopathogen *Serratia marcescens* show decreased survival compared to unexposed eggs. We next tested the immune competence of eggs under challenge from bacterial infection, and found that eggs lacked endogenous production of antimicrobial peptides, despite well-developed responses in larvae. Finally, we found that despite lacking immunity, *N. vespilloides* eggs produce an extraembryonic serosa, indicating that the serosa has lost its immune inducing capacity in this species.

Conclusions: The dependency on ephemeral resources might strongly select for fast developing animals. Our results suggest that *Nicrophorus* carrion beetles, and other species developing on ephemeral resources, face a fundamental trade-off between egg immunity and development time.

Key words: Trade-off; Burying beetle; egg immunity; developmental speed

Introduction

Exposure to harmful microbes poses numerous and diverse threats to developing animals (1). For animals with internal development, microbial pathogens that can directly harm the embryo can be controlled by the surveillance of maternal adaptive and innate immunity (2, 3). By contrast, microbial defence in animals that develop externally is provided by barrier protection from the egg surface, from maternally provided antimicrobials or through intrinsic immunity coordinated by the developing embryo (4). These modes of protection have been extensively examined in vertebrates (5). For example, avian egg shells provide direct physical protection against external microbial challenge, while mothers provision eggs prior to laying with a suite of general and specific antimicrobials, such as lysozyme, avidin and ovotransferrin (6), which provide crucial protection to the embryo prior to the maturation of the embryonic immune response. In invertebrates, parents can similarly invest in offspring defence via trans-generational immunity that provides diverse defences against pathogens and parasites that parents have encountered and which may pose specific threats to offspring (7). This can occur via deposition of antimicrobials onto the insect egg surface, or maternal provisioning of antimicrobials into the egg itself (8-13). In addition, embryos in some invertebrate species can also mount endogenous defences against pathogen challenge by producing antimicrobial peptides within eggs (9, 14, 15). However, this response not universal and is notably absent in the well-studied model species *Drosophila* (15).

Here we examine the role of egg immunity in the burying beetle *Nicrophorus vespilloides*. This species is particularly suited for this investigation because eggs of this species face considerable challenge from the bacteria they encounter during development (16). *Nicrophorus* species reproduce on small vertebrate cadavers which they bury in the soil after they are located through volatiles emitted from the carcass. Burying beetle eggs are laid into the soil adjacent to vertebrate carcasses (17). After a two-three day incubation, eggs hatch and larvae migrate to the carcass where they are communally reared by one or both parents (18). Caring parents regurgitate food to their developing larvae and also provide protection against insect competitors and predators (17, 19). In addition, parents protect offspring against bacterial competitors growing on the decomposing carcass by depositing antimicrobial secretions, e.g. lysozyme, on the carcass surface (16, 20-23). Parental lysozyme secretion

peaks during brood rearing and significantly increases larval survival (20). Larvae also contribute to brood social immunity by secreting antimicrobials that inhibit bacterial growth (24, 25). They also show a progressive increase in humoral and cellular immunity through development (26). Although different life stages of the burying beetle show both behavioral and immunological responses to reduce the negative effects of microbial challenge, studies of these responses to date have focused on post-hatch behaviors and reductions in fitness (16, 27, 28). However, pre-hatch reductions in fitness as a consequence of microbial exposure have not been studied; therefore, it remains unknown how or if eggs respond to the adverse environment in which they are laid.

In this study we investigated both the impact of soil-borne bacteria on egg development and the ability of the eggs to mount immune responses. We first measured the consequences of microbial challenge on pre-hatch fitness by assessing egg survival across contrasting environmental conditions. Next, we tested whether antimicrobial peptide genes are expressed in burying beetle eggs in response to infection (29). Briefly, we show that eggs are significantly harmed by exposure to microbes in grave soil and that eggs lack endogenous immunity. We discuss this lack of an immune response in the light of a trade-off with developmental speed.

Methods

General procedures

Experimental animals were taken from an outbred laboratory population derived from wild-caught *N. vespilloides* individuals trapped in Warmond near Leiden in The Netherlands, between May and June 2013. Beetles were maintained in the laboratory at 20°C with a 15:9 hour light:dark cycle. All adults were fed fresh chicken liver twice weekly. To collect eggs, non-sibling pairs of beetles were allowed to mate for 24 hours, after which the female was removed and provided with either a Fresh or Aged mouse carcass weighing 24-26g in a 15 cm x 10 cm plastic box filled with approximately 1-2 cm of soil. The state of found carcasses in the field across the breeding season remains unclear. Accordingly our treatments are meant to represent different extremes of the potential continuum of carcass decay. Following (16), Fresh carcasses are defined as mice that were thawed after removal from the freezer and provided directly to mated females, while Aged carcasses were allowed to age for

7 days on top of commercial peat soil before mated females were added.

Egg survival

Mated females were provided with either a Fresh (n = 35) or Aged (n = 35) carcass in order to quantify the role of carcass age on egg number and survival. Commencing the morning following set-up, boxes with mice and females were visually inspected every 12 hours to determine the timing of egg appearance. 48 hours later, eggs were removed from the soil and allowed to hatch in petri plates at 20°C containing 1.5% water agar. Egg hatch was monitored every 3 hours until no further hatching was observed.

To examine the role of soil-borne microbes on egg hatch we carried out two different experiments using a split-brood design. In the first experiment, eggs were collected from the soil from females provided with a Fresh carcass (n = 32). Each brood with a minimum of 20 total eggs (n = 30) was split into two treatment groups. Half of each brood was gently rinsed in sterile water and then allowed to hatch on sterile 1% water agar. The other half of each brood was rinsed in a solution containing the entomopathogenic bacterium *Serratia marcescens* at a density of 108/ml, after which eggs were placed to hatch onto sterile water agar. The split-brood design allowed us to control for intrinsic differences in the hatch rate of broods from different females.

In the second experiment, eggs were collected from females provided with an Aged carcass. Using a split-brood design and with the same minimum threshold for inclusion of 20 eggs (n = 29), broods were divided into two treatment groups. A control group of washed eggs from each family was transferred to sterile water agar. The other half of each brood was first surface sterilized in an antimicrobial solution of hen egg-white lysozyme (1 mg/ml), streptomycin (500 μg/ml) and ampicillin (100 μg/ml), and then placed onto water agar plates to hatch. Previous experiments have shown that eggs thus treated are free of bacteria (24).

To assess the ability of *N. vespilloides* eggs to withstand desiccation we collected eggs from soil 15 hours after females were given a carcass. This cut-off was used to ensure that eggs were roughly of the same age. Eggs were placed onto 1% sterile water agar plates and incubated for 24 hours at 20 °C.

Next, eggs were transferred to glass petri dishes and allowed to hatch at 20 °C with either 75% or 90% relative humidity (RH) in an environmental chamber. A separate set of eggs was retained on water agar as a control. The proportion of hatched eggs was scored after 3 days.

Experimental infection of *N. vespilloides* eggs and larva

To examine the capacity for eggs to mount an immune response against microbial challenge, eggs were experimentally infected with a concentrated solution of *Escherichia coli* and Micrococcus luteus. Eggs were collected 15 hours after females were provided with a fresh carcass and then kept at 20 °C for 24 hours on 1% sterile water agar. Next, eggs were pricked with a sterile 1 micron tip tungsten needle (Fine Science Tools) dipped into bacterial solution (septic injury) or with a sterile needle alone (sterile injury). After infection/sterile injury, eggs were incubated for 6 hours at 20 °C before RNA extraction. For larval infection we allowed eggs to hatch on 1% water agar. Larvae between 0-24 h old were then pricked with either a sterile needle or, with a needle previously dipped into the same bacterial solution as above. Larvae were incubated for 6 hours at 20°C before RNA extraction.

RNA extraction and real-time quantitative RT-PCR (qRT-PCR)

Total RNA of 5-10 eggs or larvae was extracted using TRIzol (Invitrogen) after which the RNA was purified and DNA digested on column with the RNeasy kit (Qiagen). The quality of the RNA preparation was confirmed spectrophotometrically. One microgram of total RNA was used for cDNA synthesis. First strand cDNA was made using the Cloned AMV First Strand Synthesis kit (Invitrogen). Each qRT-PCR mixture (25 µl) contained 2.5 ng of cDNA, and the real-time detection and analyses were done using SYBR green dye chemistry with the qPCR kit for SYBR Green I (Eurogentec) and a CFX96 thermocycler (Biorad). Thermal cycling conditions used were 50 °C for 2 min, 95 °C for 10 min, then 50 cycles of 95 °C for 15s, 60 °C for 30s, 72 °C for 30s. This was followed by dissociation analysis of a ramp from 65 to 95 °C with a read every 0.5 °C. Relative quantification for each mRNA was done using the Livak-method (30). The values obtained for each mRNA were normalized by RPL7 mRNA amount. Total RNA for each treatment was isolated twice (biological replication) and each sample was measured by qRT-PCR twice (technical replication). Comparisons between treatments (untreated, sterile

injury and septic injury) were performed within one brood.

Immune-related genes and primers used for qRT-PCR

Real-time PCR oligonucleotide primers were designed using Primer3Plus (http://primer3plus.com/cgi-bin/dev/primer3plus.cgi) by applying the rules of highest maximum efficiency and sensitivity to avoid the formation of dimers, hairpins and other artefacts. The following immune-related genes were examined: Attacin 2, Defensin 1, Defensin 2, Coleoptericin 1, Coleoptericin 2, Coleoptericin 3 and the normalizer of qRT-PCR ribosomal protein 7 (RPL7). Sequences of immune-related genes were derived from (29), and primer pairs of respective target genes were designed for qRT-PCR (Table 1).

Table 1. Primers for immune sequences of *Nicrophorus vespilloides*.

Gene	Forward primer	Reverse primer
Attacin2	5'-ACGTCACAGGAGAAGAGCTGA-3'	5'-TCGGAAGGCCTGTGTGTGTA-3'
Defensin1	5'-GTCGATACGCCCATCGGTTC-3'	5'-GCAATTGCAGACTCCGTCGA-3'
Defensin2	5'-AGAGGTGCATGCGATCTGTT-3'	5'-TGTGCCTTTGGTGTATCCGT-3'
Coleoptericin1	5'-CGAAACGGTGGTGAACAGGT-3'	5'-TGCATTGGTTGTACCGTCGG-3'
Coleoptericin2	5'-TGGTCTCCGCCGAATCCTAA-3'	5'-GCACCTGGTCTTTCGTGCTT-3'
Coleoptericin3	5'-ACTTTGGCGCGAGTCGATTT-3'	5'-TTGATCGCCCAACTCGCTTC-3'
RPL7	5'-TGCCATCAAGAAGCGCTCTG-3'	5'-GCGCTCTTGGCTTGATGGAT-3'

Embryo fixation and microscopy

The extraembryonic serosa in *Tribolium castaneum* is known to be involved in both desiccation resistance (31) and endogenous immune competence of the eggs (15). All insect species studied to date, with the exception of one group of higher flies (32, 33), develop a serosa (34). Embryonic development of *N. vespilloides* however, has not been studied. To examine the development of the serosa in *N. vespilloides*, fixed eggs were visualized under the confocal microscope (5x magnification). Eggs were placed onto 1% water agar plates at 20 °C and left for 24 hours to ensure that enough time had passed to develop the serosa. Next, eggs were fixed for 18 hours at room temperature in a solution of 4 ml phosphate buffered saline (PBS), 1 ml 37% formaldehyde and 5 ml of

heptane. They were removed from the fixative and cut in half with a scalpel. The cut eggs were washed 3 times in PBS-Tween and then stained with DAPI for 2 hours at room temperature. After staining, the eggs were washed 3 times with PBS-T and embedded in glycerol on a glass bottom petri dish. Samples were studied with a Zeiss Cell Observer.

Results

Egg number and survival is reduced in the presence of an Aged carcass

Females that were provided with an Aged carcass laid significantly fewer eggs than females that were provided with a Fresh carcass (two-tailed Mann-Whitney U Test, $P = 0.012$, Fig. 1a). In addition, the survival of eggs laid by females provided with an Aged carcass was significantly lower than the survival of eggs laid near a Fresh carcass (two-tailed Mann-Whitney U Test, $P = 0.011$, Fig. 1a). Combining these to obtain an overall estimate of brood size, by taking the product of egg number and hatch proportion, we find that broods laid near to Fresh carcasses are significantly larger than those laid near to Old carcasses (Fresh: 32.57 ± 3.01 vs Old: 23.11 ± 2.61; two-tailed Mann-Whitney U Test, $P = 0.005$). Together these data show that pre-hatch fitness is reduced by the presence of an Aged carcass.

To test the idea that bacteria in the soil cause this reduction in survival, we split broods laid near an Aged carcass and surface-sterilized one half with an antimicrobial solution while leaving the other half unsterilized. As predicted, if bacteria on the surface of eggs contributed to the failure of eggs to hatch, sterilizing eggs significantly increased egg survival when compared to washing eggs with water (paired t-test, $df = 29$, $p < 0.001$, Fig. 1b). To further examine the idea that exposure to high bacterial numbers decreases pre-hatch fitness, we again used a split-brood design and experimentally exposed eggs laid near a Fresh carcass to the soil borne entomopathogen *S. marcescens* and compared these to eggs washed in water. Exposure to *S. marcescens* had a pronounced negative effect on pre-hatch fitness (paired t-test, $df = 28$, $p < 0.001$, Fig. 1c). Notably, the reduction in survival following experimental infection, and the increase in survival following surface sterilization are roughly equivalent. Furthermore, these differences are similar to the differences first observed in untreated eggs laid near Aged and Fresh carcasses. Together, these data strongly indicate that harmful bacteria in the environment of Aged carcasses significantly reduce pre-hatch fitness.

Antimicrobial peptide expression in response to infection

Although survival of *N. vespilloides* eggs is reduced in the presence of an Aged carcass, overall egg viability is still quite high; approximately 70% of the eggs still survive even under these challenging conditions (Fig. 1a). As we have previously shown that the eggs of *Tribolium castaneum* can induce antimicrobial peptide genes upon infection (15), induction of antimicrobial peptides might also increase survival in adverse conditions for the eggs of *N. vespilloides*. We measured gene expression of several antimicrobial peptides after both sterile injury and septic injury in *N. vespilloides* eggs and larva. Surprisingly, in eggs we found marginal, if any, upregulation of antimicrobial peptide genes after infection (Fig. 2). Only one gene (Coleoptericin 2, Fig. 2e) was induced over 10 fold after infection. By contrast, freshly emerged larvae show clear induction of all antimicrobial peptide genes tested (Fig. 2). To verify that mRNA levels are lower in the eggs, we compared infected eggs with infected larvae. As expected, transcript levels are higher in larvae (Supplementary Fig. 1). These data show that although freshly emerged larvae can induce immune genes upon infection, eggs of *N. vespilloides* show very limited AMP inducing capacities.

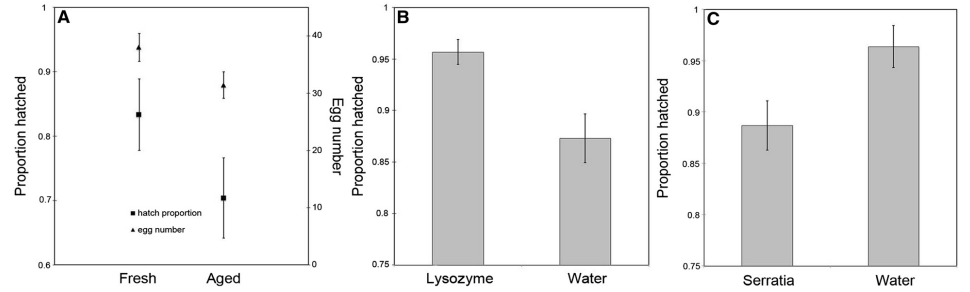

Figure 1 Egg survival and number under different treatments. A) Both egg number and egg survival are significantly lower when in the presence of an Aged carcass. B) Eggs collected from an Aged carcass show increased survival when sterilized, indicating the negative effect of high bacterial numbers surrounding Aged carcasses. C) Eggs collected from a Fresh carcass show decreased survival when experimentally exposed to the entomopathogenic bacterium *S. marcescens*.

Eggs develop an extraembryonic serosa

The immune response of *Tribolium castaneum* eggs depends on the presence of an extraembryonic epithelium called the serosa (15). By contrast, the immune response is poor in eggs of the fruit fly *Drosophila melanogaster* which lack this epithelium. Given the apparent absence of endogenous egg immunity in *N. vespilloides*, we hypothesized that this species, like *Drosophila*, would lack a serosal epithelium. We tested this idea in two ways, first by measuring desiccation tolerance of eggs, as the serosa imparts drought resis-

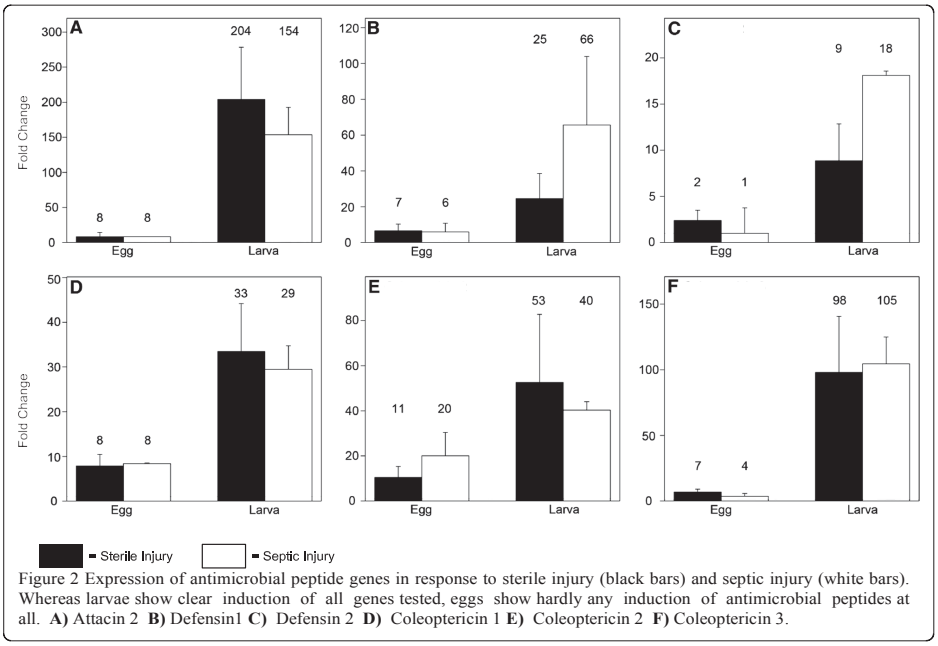

Figure 2 Expression of antimicrobial peptide genes in response to sterile injury (black bars) and septic injury (white bars). Whereas larvae show clear induction of all genes tested, eggs show hardly any induction of antimicrobial peptides at all. **A)** Attacin 2 **B)** Defensin1 **C)** Defensin 2 **D)** Coleoptericin 1 **E)** Coleoptericin 2 **F)** Coleoptericin 3.

tance in *T. castaneum* (31), and second by directly examining DAPI stained eggs via confocal microscopy. *N. vespilloides* eggs are highly susceptible to desiccation; egg survival dropped from 92% at 90% RH to 0% at 75% RH (chi-square test, $p < 0.001$). Although this result, together with the absence of endogenous immunity is consistent with the absence of a serosal epithelium, DAPI-stained confocal microscopy clearly revealed an epithelium around the egg (Fig. 3a). This epithelium could easily be distinguished from the amnion in optical sections (Fig. 3b) and was identified as serosa.

Discussion

Nicrophorus eggs are deposited into the soil in close proximity to vertebrate carcasses (17). Eggs in this grave-soil environment are exposed to increased nutrient fluxes from carcass decay that increases the biomass of endogenous bacteria and of bacteria that migrate to the soil from the perforated carcass (35). Several previous studies have documented the diverse and persistent negative effects of this flora on the survival and growth of developing larvae (16, 27, 28, 36). Here we extend these findings by showing that carcass associated bacteria also significantly reduce the survival of *Nicrophorus* eggs. We found that

females provided with an Aged carcass laid fewer eggs that were less likely to hatch than their female counterparts provided with a Fresh carcass (Fig. 1a). In addition, we show that egg hatching success is a direct function of bacterial exposure; eggs washed free of surface-associated bacteria show increased survival compared to unwashed eggs (Fig. 1b) while eggs washed in a bacterial solution show decreased survival compared to unexposed eggs (Fig. 1c). The overall consequence of this exposure is an approximate 30% decline in potential brood size. This cost, in addition to those already identified at later stages of beetle development, clarify the risks to *Nicrophorus* of rearing young on microbe-rich contaminated carcasses.

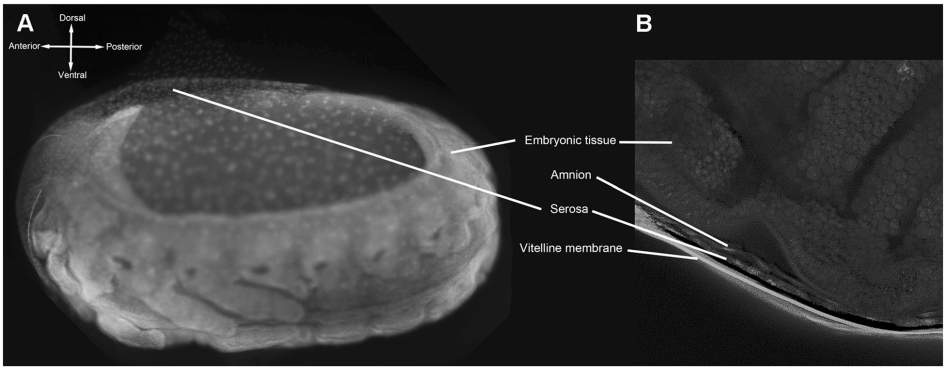

Figure 3 The eggs of *N. vespilloides* develop an extraembryonic serosa. **A)** Overview of a complete embryo, the developing head is visible at the anterior. The serosal epithelium can be clearly seen just above the head. **B)** Optical section of an *N. vespilloides* egg. The embryo, serosa, amnion and vitelline membrane can be clearly distinguished.

The carcasses that Nicrophorus larvae rely upon are classical bonanza sources that are unpredictable in time and space. Parents modify the carcass in numerous ways that increase larval growth and survival. The carcass is buried, stripped of fur and coated with both antibacterial and antifungal compounds (20, 22, 23, 27, 37). In addition, parents defend the carcass before and following the arrival of larvae from insect competitors like flies or other carrion beetles (16, 17, 38). In contrast to these elaborate behaviours used to defend larvae, there is surprisingly little direct evidence for parental defence of eggs. Earlier research failed to find any lysozyme-like activity inside or on the *N. vespilloides* egg (24), suggesting an absence of direct antimicrobial provisioning. And although antiseptic volatiles secreted by parents into the soil surrounding the carcass may provide an indirect benefit to eggs, this is as yet untested (37).

Why is egg defence apparently missing in this species? One possible explanation is that explicit care of eggs trades-off with carcass maintenance

and defence. Thus rather than investing in individual eggs, parents instead invest in preserving the resource that will provide an aggregate benefit to any larvae that survive the egg-stage and eventually migrate to the carcass. Consistent with this idea, egg production in *Nicrophorus* does not appear to engender significant costs (39), the number of eggs observed in experimental *Nicrophorus* broods typically exceeds the number of larvae found on the carcass and infanticidal culling is common (40, 41). It is likely that there is further mortality in the field where eggs face additional predation risks that are not present in the lab. Finally, by excreting antimicrobials on the carcass surface, parents can maintain the carcass in a suitable state for extended time periods, assuming it is found prior to significant decomposition. Also, because parents prefer a Fresh over an Aged carcass (16), eggs may not have been selected to be able to cope with high levels of associated bacteria on extensively decomposed carrion.

A second possibility is that explicit defence is prohibitively expensive, especially when, even in its absence, egg survival is quite high (Fig. 1c). This contrasts markedly with other species, like earwigs, where untended eggs challenged with mold infection show far more dramatic declines in hatch success (42). Although we do not know the cause for high rates of intrinsic survival, it is possible that this is facilitated by the barrier defence provided by the embryonic serosa (Fig. 3). If so, this would be consistent with an immune-related function for the *Nicrophorus* serosa, even if the serosa in this species appears not to extensively regulate endogenous AMP production as it does for eggs of *Tribolium castaneum*. A challenge for future studies is to explicitly test this hypothesis using RNAi based targeted knock-outs of the developmental genes that regulate the production of this extraembryonic tissue.

Even in the absence of parental protection, eggs of some insects retain the capacity to generate an endogenous immune response against pathogen challenge (9, 14, 15); this is thought to be one important cause for the low incidence of parental care in insects (43, 44). Yet this endogenous response is absent in *N. vespilloides*. In that respect, there are striking similarities in development beween *N. vespilloides* and *D. melanogaster*. Both species lack inducible egg immunity and develop on ephemeral resources that favour rapid development times (15, 17, 45), and specifically rapid embryonic development. Embryonic development in *Nicrophorus* is approximately 3-6 times faster than *Tribolium* and Manduca (46, 47), and about 20 hours faster than Aedes, which

are known to go into diapause, meaning they have to survive for a long time until the conditions favour hatching (48). By contrast, *Nicrophorus* develop in the presence of a highly valuable and decaying resource; individuals need to hatch, feed and disperse before the carcass is either claimed by another animal or becomes unsuitable for development. This strong selection for fast development might be reflected by a trade-off between a well-protected but slow developing egg and a fast-developing but less protected egg. Similar trade-offs between growth and immune competence are known from plants (49), birds (50) and insects (51, 52). Although additional experiments are needed to confirm the relation between rapid development and the lack of immune competence in insect eggs, the high survival and poor immune competence of both *N. vespilloides* and *D. melanogaster* eggs under normal conditions suggests that fast development is obtained at the expense of immune competence.

Conclusions

Our work builds upon previous studies demonstrating the profound costs to *N. vespilloides* from rearing their offspring in the presence of microbial competitors or pathogens in the soil environment. Although parental care in this species can serve to mitigate some of these risks, our data suggest that at least direct care does not extend to eggs. The indirect effects of fumigation with volatiles of the surrounding microhabitat might be important, however this conjecture requires further testing. The lack of direct parental provisioning of eggs may result from a trade-off between egg protection and carcass maintenance. Similarly, the lack of immune competence may be caused by a trade-off between immunity and the need for rapid growth on a rich and ephemeral resource. Although similar life-history trade-offs are known in a broad range of species, we are unaware of results showing this trade-off for eggs. This result therefore has broad implications owing to the obvious importance of egg survival for lifetime reproductive success, and suggests the need to investigate the development of immune competence more broadly as a function of developmental timing.

Competing interests

The authors declare that they have no competing interests.

Author contributions

DER, CGCJ and YW conceived of the study. YW and CGCJ carried out all experimental work on survival, desiccation resistance, response to infection and qRT-PCR. HV and AV designed primers for qRT-PCR. CGCJ and MVDZ performed the microscopy. YW, CGCJ and DER wrote the initial draft of the paper. DER, CGCJ, YW, HV, MVDZ and AV analysed and discussed data and their interpretation, and helped draft the final manuscript. All authors read and approved the final manuscript.

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Data accessibility

All data used in this manuscript are present in the manuscript and its supporting information.

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Supplementary information

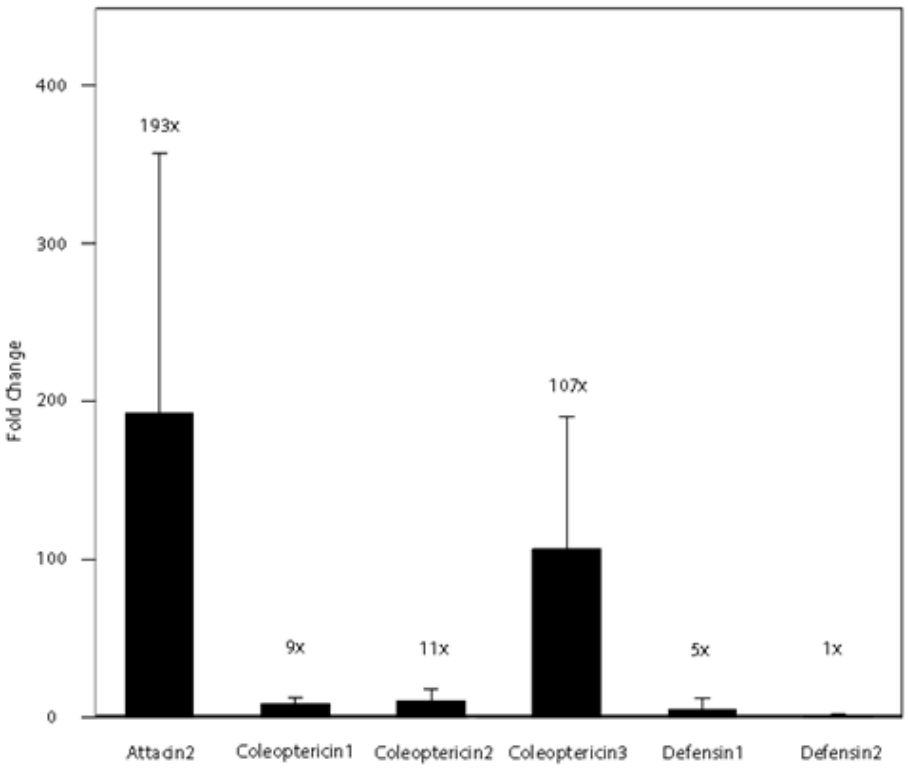

Figure S1. Expression differences between infected eggs and infected larvae. Expression of antimicrobial peptides in general is significantly higher in larvae than in eggs. Only the difference in Defensin2 was small, which is expected as it is the least induced antimicrobial peptide in this study.

Chapter 3

Colonization and transmission of the gut microbiota of the burying beetle, *Nicrophorus vespilloides*, through development

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Abstract

Carrion beetles in the genus *Nicrophorus* rear their offspring on decomposing carcasses where larvae are exposed to a diverse community of decomposer bacteria. Parents coat the carcass with antimicrobial secretions prior to egg hatch (defined as Pre-Hatch care) and also feed regurgitated food, and potentially bacteria, to larvae throughout development (defined as Full care). Here we partition the roles of pre- and post-hatch parental care in the transmission and persistence of culturable symbiotic bacteria to larvae. Using three treatment groups (Full-Care, Pre-Hatch care only, and No Care), we found that larvae receiving Full-Care are predominantly colonized by bacteria resident in the maternal gut, while larvae receiving No Care are colonized with bacteria from the carcass. More importantly, larvae receiving only Pre-Hatch care were also predominantly colonized by maternal bacteria; this result indicates that parental treatment of the carcass, including application of bacteria to the carcass surface, is sufficient to ensure symbiont transfer even in the absence of direct larval feeding. Later in development, we found striking evidence that pupae undergo a aposymbiotic stage, after which they are recolonized at eclosion with bacteria similar to those found on the moulted larval cuticle and on the wall of the pupal chamber. Our results clarify the importance of pre-hatch parental care for symbiont transmission in *Nicrophorus vespilloides*, and suggest that these bacteria successfully outcompete decomposer bacteria during larval and pupal gut colonization.

Importance: Here we examine the origin and persistence of the culturable gut microbiota of larvae in the burying beetle *Nicrophorus vespilloides*. This insect is particularly interesting for this study because larvae are reared on decomposing vertebrate carcasses where they are exposed to high-densities of carrion-decomposing microbes. Larvae also receive extensive parental care in the form of carcass preservation and direct larval feeding. We find that parents transmit their gut bacteria to larvae both directly, through regurgitation, and indirectly via their effects on the carcass. In addition, we find that larvae become aposymbiotic during pupation, but are recolonized apparently from bacteria shed onto the insect cuticle before adult eclosion. Our results highlight the diverse interactions between insect behavior and development on microbiota composition. They further suggest that competitive interactions mediate the bacterial composition of *Nicrophorus* larvae together with, or apart from the influence of beetle immunity, suggesting that the bacterial communities of these insects may be highly coevolved with their host species.

Key words: *Nicrophorus*, parental care, symbiosis, microbiota, transmission

Introduction

Animals are colonized by a diverse community of bacterial symbionts that play crucial roles in their ecology and evolution (1–3). This has been especially well studied in insects, whose bacterial symbionts can influence traits ranging from mate and diet choice (4, 5) to susceptibility to natural enemies (6, 7). Bacterial symbionts can also differ in the fidelity of their associations with their insect hosts. Endosymbionts like *Buchnera* in aphids, that serve obligate functions for their insect hosts by overcoming host nutritional deficiencies, are highly specific and have been aphid-associated for millions of years (8). At the opposite extreme, insects can retain transient associations with bacteria whose effects are more variable (5, 9–11). Although different factors may underlie the divergent influences of bacterial symbionts on insect hosts, one key component is the way that bacteria are transmitted between insect generations (12). Whereas obligate symbionts are always transmitted vertically, often via direct passage through eggs, more transient associations, typical of the gut microbiota, involve an external stage where bacteria are reacquired horizontally each generation via ingestion (13, 14).

Distinguishing symbionts on the basis of transmission mode (vertical versus horizontal) has been extremely useful by focusing attention on how this can align the fitness interests of symbionts and hosts (15, 16). However, many associations between insects and their microbial symbionts fall somewhere in the middle of these strict extremes. Among diverse possibilities, trophallaxis and coprophagy occurs when bacteria are passed horizontally between individuals via oral-oral/anal contact or fecal consumption (17–19). Similarly, horizontal symbiont transmission can take place via ingestion of the bacteria-smeared egg-coat or via consumption of bacteria-rich capsules (20, 21). While these methods of transfer can effectively vertically transmit symbionts from parent to offspring (13), the presence of an environmental component implies that young and developing insects can be simultaneously colonized by beneficial symbionts as well as environmental bacteria that can harm the host (21, 22). In these cases, establishment of the inherited microbiota will be partly dependent on the ability for inherited symbionts to competitively exclude environmental bacteria, as well as the timing and manner of their acquisition (23, 24). Additionally, especially for holometabolous insects that undergo a complete metamorphosis, the manner of acquisition can change markedly throughout development, at one stage occurring from the mother while at later stages potentially through alternative transmission routes (12,

13).

Here, we examine the mechanisms of transmission and stability of the culturable gut microbiota of the carrion beetle, *Nicrophorus vespilloides*, throughout its development. This system is particularly interesting for addressing these questions given the peculiar life-history of these organisms. *Nicrophorus* beetles are reared on decomposing carrion where they encounter and ingest high densities of microbes (25–29). Eggs are laid in the soil near to the carcass (25). Upon hatching larvae migrate to the carcass where they both self-feed and are fed regurgitated material from the caring parents (25, 30, 31). Next, following a ~ 6-7 day feeding period upon the carcass, larvae cease feeding and disperse into the surrounding environment where they eventually pupate individually in underground chambers. Finally, pupae eclose into adults, and emerge from the pupal chambers to commence feeding (25, 32).

N. vespilloides larvae may be exposed to a varied microbiota throughout development, and this will likely be influenced by both the presence of parents and the stage of development (26–29, 33). First, parents may modify the carcass microbiota by coating it in antimicrobial secretions throughout the period of parental care (22, 27, 34). Notably, these secretions are not sterile and contain significant numbers of bacteria that can proliferate on the carcass (29). Secondly, parents feed larvae with regurgitated food which may facilitate the transfer of the parental gut microbiota to offspring (Post-hatch care) (31). Finally, following dispersal, larvae cease feeding, thereby preventing continued colonization from diet-borne bacteria; and then during metamorphosis they shed the larval gut (25). At present, there is no understanding of the dynamics of these gut bacterial communities through time.

There is little knowledge of the colonization dynamics of *Nicrophorus* gut bacteria or the extent to which colonization is influenced by parental care, a hallmark of this system. To examine these questions we manipulated *N. vespilloides* parental care and used a culture-based approach to monitor the dynamics of symbiont colonization and stability through development. Although culturing can underestimate bacterial densities when compared to total cell counts or sequence-based approaches (see Supplemental Figure 1), this approach allowed us to examine the largest set of experimental conditions, while also identifying the bacterial groups that can be experimentally manipulated to understand mechanisms of colonization and community

assembly of the microbiota using the *Nicrophorus* model system. Briefly, our results provide strong evidence that beetle parents play a defining role of the establishment of the bacteria residing in *Nicrophorus* larval guts; however, continuous parental care and feeding is not essential for the stable maintenance of this microbiota. We also find that pupae undergo an aposymbiotic stage, after which they are recolonized by bacteria in the pupal chamber. We discuss these results in the context of the role of the *Nicrophorus* microbiota for beetle fitness.

Methods

General procedures

Experimental beetles were taken from an outbred laboratory population derived from wild-caught *N. vespilloides* individuals trapped in Warmond near Leiden in The Netherlands, between May and June 2014. Beetles were maintained in the laboratory at 20 °C with a 15:9 hour light:dark cycle. All adults were fed fresh chicken liver twice weekly. To generate outcrossed broods, non-sibling pairs of beetles were allowed to mate for 24 hours in small plastic containers with soil. Next the mated pair were provided with a freshly thawed mouse carcass weighing 24-26 g in a 15cm × 10cm plastic box filled with approximately 1-2 cm of moist soil. Although fresh carcasses may differ in bacterial composition from aged carcasses (26, 29), our use of fresh carcasses in this study ensured higher brood success and is consistent with recent data showing that most mouse carcasses are discovered by burying beetles shortly after they are placed in experimental forests (35). Broods were reared in sterile soil until the point of larval dispersal from the carcass, after which larvae were transferred to new boxes for pupation with unsterilized peat soil to complete development. Soil was sterilized using two autoclave cycles at 121 °C for 30 minutes in a volume of 160 Liters (Tuttnauer 5075 ELV), with a cooling interval between cycles.

Maternal care manipulation

To examine the role of parental care on the acquisition and composition of beetle gut bacteria, we reared larvae under three treatment conditions that modified the degree of parental care they received (26, 30): 1) Full Care (FC)

broods experienced complete parental care, including pre- and post-hatch care; 2) Pre-hatch parental care (PPC) broods were reared on a carcass that had been prepared by the female, after which she was removed prior to the hatch/arrival of larvae; and 3) no-care (NC) broods experienced neither pre- nor post-hatch care. Broods in all treatments were initiated similarly. Mated females were provided with a fresh carcass and induced to lay eggs. Eggs were collected and surface sterilized within 12-24 hours and these were then used to generate replicate broods of 15-20 larvae each. Females remained with their prepared carcasses in FC broods, while females were removed prior to reintroducing larvae in the PPC broods. NC larvae were provided with a freshly thawed carcass with a sterile incision in the abdomen to permit larval entry.

Bacterial density and composition throughout development

We examined the dynamics of *N. vespilloides* intestinal microbiota through time by destructively sampling beetles throughout development. To quantify gut bacterial CFU, the whole intestinal tract from each beetle (n = 3 at each time point) from independent broods was carefully removed with fine forceps and suspended in 0.7 ml sterile sodium phosphate buffer (PBS; 100 mM; pH 7.2); beetles were surface sterilized by 75% ethanol and PBS solution (100 mM; pH 7.2) prior to gut dissection. The inner contents of pupa were examined in their entirety owing to the absence of a clear gut at this stage. Because 0.1 mL were plated from 0.7 mL dilutions, our limit of detection is ~ 10 cells/larval gut. Newly eclosed adults were unfed prior to sampling. Individual gut/pupal contents were serially diluted in PBS and plated on 1/3 strength Tryptic Soy Broth agar and incubated at 30 °C. To directly compare bacterial densities determined from total microscopic counts versus via CFU from plating, bacterial cells per beetle gut (n = 5 from each time point) from independent broods with Full Care were quantified on 1/3 TSA agar and by estimating total cell numbers via Fluorescence-based microscopy. Microscopic counts were further partitioned into Live and Dead cells with LIVE/DEAD staining using SYTO9 and propidium iodide (BacLight Bacterial Viability Kit, Invitrogen). Samples were observed under a Zeiss Axioplan 2 Imaging microscope. The filters used were 470/40 (green) and 572/25 nm (red) for excitation and 525/50 (green) or 629/62 nm (red) for emission. Three images were counted for each sample at each dilution. Although plating for CFU consistently underestimates bacterial densities, this approach recovered up to 60% of total counts and the

dynamics of bacterial densities perfectly mirror those based on total counts. The composition of the maternal microbiota was characterized from n = 3 mated females.

At each time point from each treatment, we isolated random colonies (n ≥ 100) on 1/3 TSA from individual beetles to analyze for species identification using MALDI-TOF Mass Spectrometry (Matrix Assisted Laser Desorption Ionization-Time of Flight) with the Biotyper platform (Bruker Daltonic GmbH). By generating unique whole-cell protein-based fingerprints for each colony, the Biotyper permits highly reproducible identification of bacterial colonies to the genus or species level. Because of its reproducibility, ease of use and cost effectiveness, the Biotyper is used extensively in clinical and public health microbiological laboratories (36) and is finding increased use in ecological studies (37–39). To standardize growth prior to analysis, individual colonies were tooth-picked onto a 1/3 TS plate and grown overnight. Colonies were then transferred directly to a 96-well steel MALDI-TOF target plate and coated with 1 μl of alpha-cyano-4-hydroxy cinamic acid (HCCA) matrix comprised of Acetonitrile (50%), Trifluoroacetic acid (2.5%) and water (47.5%), and dried at room temperature. The target plate was subsequently inserted into the Biotyper system for analysis. Next, mass spectrometry was carried out using the MALDI Biotyper RTC (Realtime classification) and analyzed using Biotyper 3.0 (Bruker DAltonic GmbH). Spectra were collected under the linear positive mode in the mass range of 3 to 20 kDa and a sample rate of 0.5 GS/s (laser frequency, 60 Hz; ion source 1 voltage, 20.08 kV; ion source 2 voltage, 18.6 kV; lens voltage, 7.83 kV). The Bruker bacterial test standard (BTS 8255343) was measured for standardization of MALDI calibration before the specimens were processed. Spectra were compared to the reference library provided by Bruker which identified 62.3% of the colonies to species level overall using a stringent cut-off of 1.699, below which indicated no reliable identification (in the Bruker library) (40, 41). To confirm these assignments and to establish the identity of colonies whose spectra were not included in the Bruker database, all unique MS spectra (including both those with positive hits and those not present in the Biotyper database) were subsequently analyzed using 16s rRNA sequencing. Colony PCR using primers 27F (5'AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') was used for bacterial 16s rRNA gene amplification (42). The PCR cycling conditions were as follow: 95 °C for 5 min, then 34 cycles of 95 °C for 45 s, 55 °C for 45 s, 72 °C for 1 min. PCR products were directly sequenced via the DNA Markerpoint in Leiden and 16s rRNA gene sequences

were classified for bacterial taxonomy using a nucleotide BLAST against the NCBI database. The Bruker database was manually updated to include new samples thus obtained.

A second experiment was conducted to determine the source of bacterial re-colonization following beetle pupation. Pupae were removed from their soil chambers and both the inside wall of the soil chamber and the cuticles of the pupae were swabbed with a sterile, moist, cotton swab. The bacteria on the swab were resuspended in sterile water and serially diluted onto 1/3 TS agar. Finally, soil from outside the pupal chamber was collected and diluted into PBS and plated. Colonies were isolated and identified as above using a combination of MALDI-TOF Biotyping and 16s rRNA sequencing. To exclude rare or transient bacterial species, we established a minimum threshold frequency of 1%, averaged over all sampling periods for each treatment set, prior to analysis of community composition.

Statistical analysis

Bacterial CFU through time were analyzed using General Linear Models (GLM) with time and treatment as factors. Community composition was analyzed using the Vegan package in R (43). Beta diversity among the different treatments was analyzed using ANOSIM, which is based on a Bray-Curtis dissimilarity matrix (44, 45). The R function betadisper was used along with ANOSIM to test for equal dispersion between groups. Analyses of dispersion between parental care treatments and larvae detect no significant differences between groups ($F = 1.796$, $P = 0.12$, Number of permutations = 199), nor did we detect any differences in dispersion between any of the comparisons of microbial communities examined following microbial recolonization of pupae (all tests > 0.05). Dendrograms to examine community similarity were generated based on the matrix of mean within-group and between-group distances and the R function hclust was used for hierarchical clustering.

Results

Bacterial CFU vary through development and as a function of parental care

The CFU of intestinal bacteria was quantified throughout development

for three treatment groups corresponding to different levels of parental care. Following hatching from sterile eggs, larvae from all treatments rapidly acquire high bacterial densities within their guts. Bacterial densities vary significantly through time (GLM analyses: df = 10, P < 0.001) and as a function of treatment (GLM: df = 2, P = 0.006) and vary across nearly 6 orders of magnitude as a function of developmental stage. During larval feeding on the carcass, bacterial densities increase in all treatments, reaching densities of ~ 106 to 107 / larva. By contrast, following dispersal, bacterial populations precipitously decline until, during pupation, bacteria were undetectable. Finally, as pupae eclose and reemerge from pupal chambers, they reacquire a high-density bacterial population within their guts (Fig. 1B/C). It is notable that this recovery occurs prior to feeding and before emergence from the pupal chamber, indicating that recolonization takes place from bacteria resident within the pupal chamber itself. The dynamics of colonization are broadly insensitive to experimental methods, as estimates of density based on total microscopic counts perfectly mirror those determined by plate counting (Fig. 1C), although CFU based estimates consistently underestimate Live counts (Table S2). Minor differences were observed between total and live cell counts, although these are only significantly different at days 3 and day 12, accounting for 43% and 28% of total cell numbers, respectively (paired t-tests, df = 4, Day 3: p = 0.039; Day 12: p = 0.019).

Composition of *N. vespilloides* larval symbionts

Although bacterial densities differ across parental-care treatments there is broad overlap in the dynamics of CFU change through time. Despite these similarities, the composition and diversity (Table S1) of these communities may vary. To understand these differences and to illuminate transmission dynamics from mothers to larvae, we tracked community composition of gut bacteria within larvae throughout development (Fig. 2) using MALDI-TOF Mass-Spectrometry and compared these to the maternal samples. The maternal microbiota was dominated by four bacterial genera that together comprised > 65% of recovered CFU, including *Providencia*, *Morganella*, *Vagococcus* and *Proteus*, with several other genera appearing in lower frequencies (Fig. 2). We next examined genus level composition across the three larval treatment groups by ANOSIM. As anticipated if transmission occurs via parents, we observed significant overlap in the bacterial communities of parental and larval gut communities from larvae receiving parental care throughout development

($R_{FC\text{-}g\ vs\ Mother}$=0.277; P=0.028, Table 1), as R values < 0.25 correspond to "barely separable" groups (46). Equally, although to a lesser degree there is concordance between the maternal microbiota and those of larvae receiving pre-hatch care only ($R_{PPC\text{-}g\ vs\ Mother}$= 0.331; P = 0.066, Table 1). By contrast, larvae reared in the absence of parental care are highly diverged from the parental microbiota ($R_{NC\text{-}g\ vs\ Mother}$= 1; P = 0.007, Table 1) (Figs. 3A and 3B). In particular, the gut community of NC larvae was shifted towards bacterial groups likely acquired from either the soil or the carcass (Figs 2 and 3C), e.g. *Escherichia coli* (23.5%), *Serratia* (20.4%) and *Staphylococcus* (19.2%).

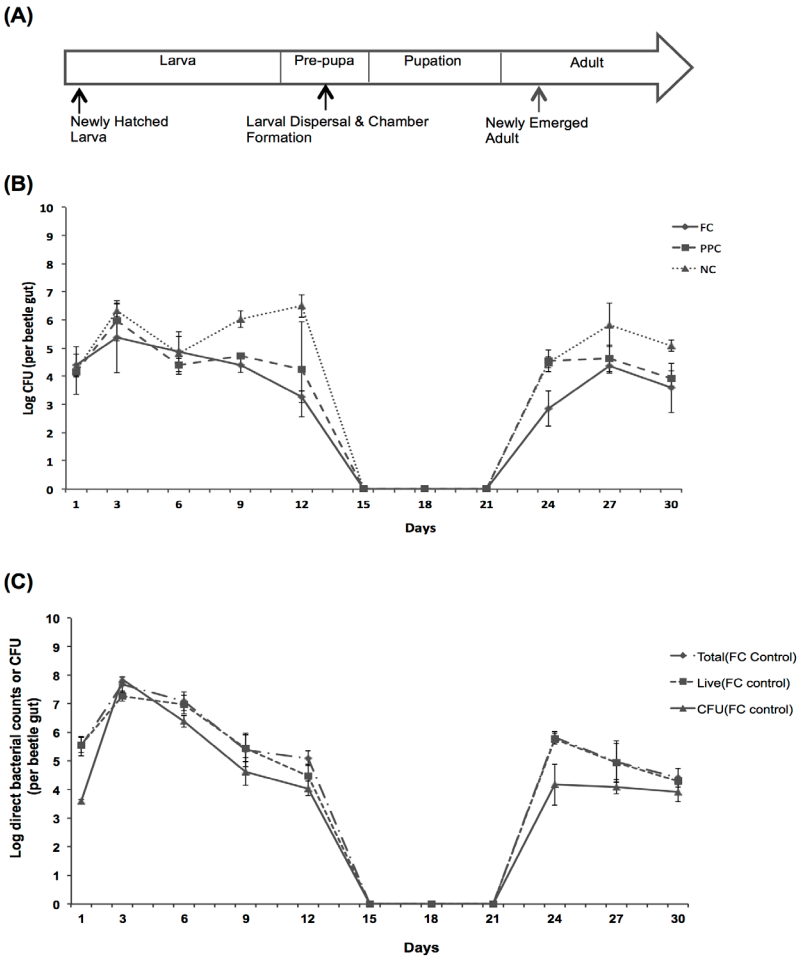

Figure 1 Change in total cell, live cell, and CFU of *Nicrophorus vespilloides* gut bacteria throughout development. (A) Overview of the time course of beetle developmental. (B) Change in CFU of host gut contents through time

(means ± standard deviation (SD); n = 3/time point). FC corresponds to larval gut samples from full parental care broods, PPC corresponds to gut samples from preparental care broods, and NC corresponds to larval gut samples from no care broods. (C) Counts of total cells, live cells, and CFU in samples from FC broods (means ± SD; n = 5/time point). The limit of detection is ~10 cells/gut sample.

In comparing the larval microbiota of the three treatment groups, ANOSIM analysis illustrated clear differences between the treatment groups overall (Global test: R = 0.815, P = 0.001) and although there are differences between the FC and PPC larvae, there is much greater similarity between the two groups with parental care ($R_{FC\text{-}g \text{ vs } PPC\text{-}g}$ = 0.665; P = 0.001) compared to either care group and the no-care larvae ($R_{FC\text{-}g \text{ vs } NC\text{-}g}$ = 0.956, P=0.001; $R_{PPC\text{-}g \text{ vs } NC\text{-}g}$ = 0.994, P=0.001) (Table 1, Fig. S1). This is also apparent in the Venn diagrams in Fig. 3A, focusing on presence/absence of specific bacterial groups. Together, these results indicate that transmission of the beetle microbiota occurs predominantly from parents to offspring. However, they also reveal that continued replenishment of bacteria from parent to offspring via feeding is unnecessary to establish the endogenous microbiota. Instead transmission can occur indirectly via deposition of the maternal bacteria on to the carcass by the mother during carcass preparation and subsequent colonization of larva via self-feeding.

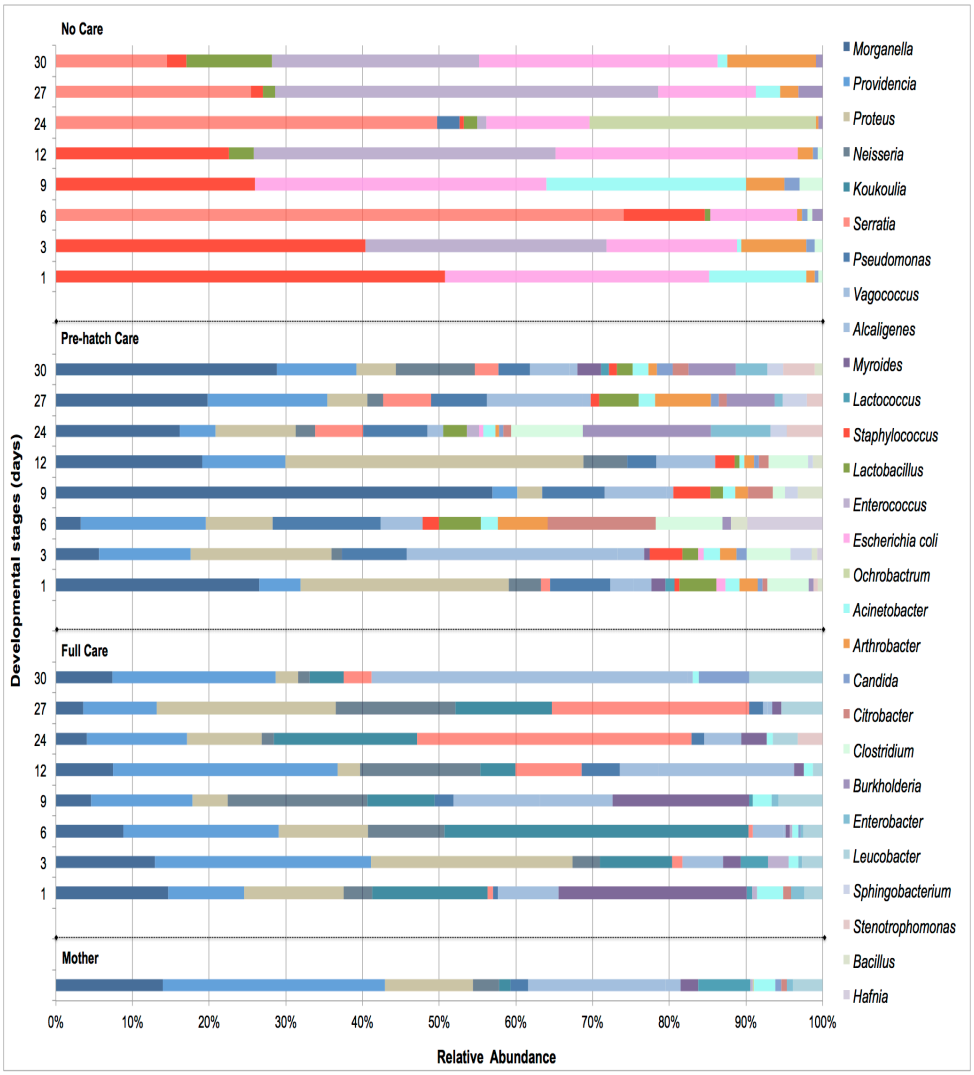

Figure 2 Composition of *N. vespilloides* gut microbiota throughout development. The maternal gut microbiota is shown at the bottom while treatment designations are the same as in Fig. 1. No CFU were detectable between days 15 to 21 of larval development, corresponding to the duration of pupation. Three individual larvae were independently analyzed for each time point. The y axis of day 1 to day 9 refers to the larval stage, day 12 corresponds to the pre-pupal stages, and day 24 to day 30 refers to adult formation.

Table 1 ANOSIM analysis on bacterial community dissimilarity. Subscripts correspond to the site of isolation; e.g. FC-g corresponds to gut samples, FC-c corresponds to the pupal carapace and the wall of the pupal chamber, and Soil corresponds to bulk soil outside the pupal chamber.

Groups	R statistic	Significance (P value)	Number of permutations	Number of observed	Test model
FC-g, PPC-g, NC-g	0.8152	0.001	999	352	Global
FC-g, PPC-g, NC-g, Mother	0.741	0.001	999	264	Global
FC-c, PPC-c, NC-c, Soil	0.7493	0.001	999	814	Global
FC-g, NC-g	0.9556	0.001	999	144	Pairwise
PPC-g, NC-g	0.9939	0.001	999	64	Pairwise
FC-g, PPC-g	0.6651	0.001	999	144	Pairwise
FC-g, Mother	0.2769	0.028	999	24	Pairwise
PPC-g, Mother	0.3306	0.066	999	24	Pairwise
NC-g, Mother	1	0.007	999	24	Pairwise
FC-g, FC-c	0.3177	0.068	999	54	Pairwise
PPC-g, PPC-c	0.0242	0.052	999	54	Pairwise
NC-g, NC-c	0.0134	0.397	999	54	Pairwise
FC-c, Soil	0.8889	0.094	720	9	Pairwise
PPC-c, Soil	1	0.114	720	9	Pairwise
NC-c, Soil	1	0.099	720	9	Pairwise

Re-colonization of *N. vespilloides* symbionts

An important result from these analyses is the aposymbiotic stage occurring during pupation, followed by recolonization from within the pupal chamber. Notably, this result based on CFU was further confirmed by direct microscopic counts (Fig. 1C). To assess the source of recolonization, we sampled bacterial populations from the pupal cuticle and the wall of the pupal chamber, together with samples from the bulk soil in which pupal chambers were constructed. Treatment designations are as above, with the addition of subscripts corresponding to each sampling site. For example, FC-g refers to samples taken from the guts of larvae receiving Full Care, while FC-c represents samples from the cuticle and chamber wall of these same larvae. These analyses showed that the *N. vespilloides* pupal cuticle and chamber soil had very similar compositions (FC-g, FC-c: R = 0.32, P = 0.068; PPC-g, PPC-c: R = 0.02, P = 0.052; NC-g, NC-c: R = 0.03, P= 0.0397 by Pairwise test of ANOSIM, Table 1), and that these were diverged compared to the bulk soil (FC-c, Soil: R = 0.89, P = 0.094; PPC-c, Soil: R = 1, P = 0.114; NC-c, Soil: R = 1,

P = 0.099 by Pairwise test of ANOSIM, Table 1). Importantly, many bacterial

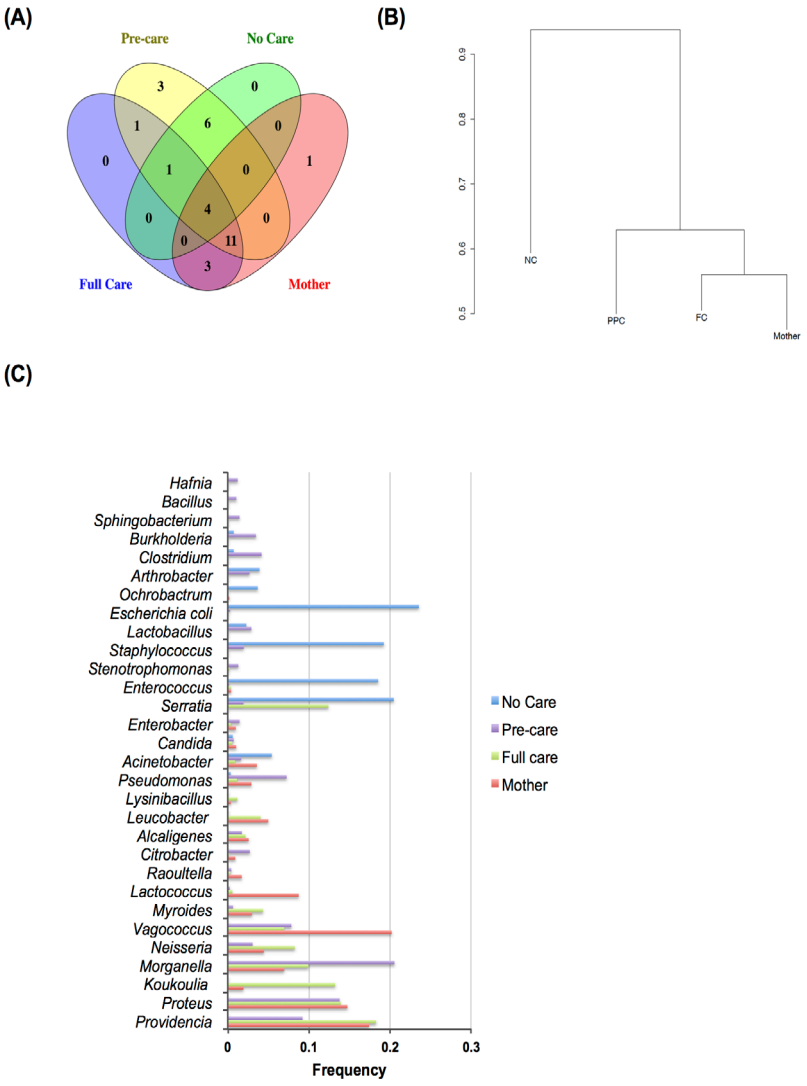

Figure 3. Frequencies of bacteria from gut communities across parental care treatments. (A) Shared and unique genera between treatment groups. Strains with a minimum frequency of 1% were included. (B) Hierarchical clustering on mean similarity of gut microbiota between treatment groups. (C) Overall composition of gut communities across treatments. Strains with frequencies lower than 1% across all communities were excluded from plots.

genera irrespective of treatment, were found in the pre-pupal gut and the cuticle but infrequently or not at all in the soil. For example, the most common bacterial groups in FC larvae contained *Providencia* (FC-g: 18.3% vs FC-c: 17.1%), *Morganella* (FC-g: 10.0% vs FC-c: 8.7%), *Proteus* (FC-g: 14.0% vs FC-c: 3.9%), *Vagococcus* (FC-g: 7.0% vs FC-c: 6.1%), *Neisseria* (FC-g: 8.3% vs FC-c: 4.7%), and *Koukoulia* (FC-g: 13.2% vs FC-c: 8.9%), while these were undetected in soil. Similarly, the most abundant genera in NC beetles were only found in NC-g and NC-c: *Escherichia coli* (NC-g: 23.5% vs NC-c: 23.8%), *Enterococcus* (NC-g: 18.5% vs NC-c: 18.8%) (Fig. 4A and 4B).

These results indicate that the core components of previously colonized gut bacteria can successfully recolonize the host intestinal system after the aposymbiotic stage characteristic of pupation. Thus, although transmission and recolonization to larvae may occur via the environment, the bacterial species that recolonize the newly eclosing adult are highly biased towards bacterial species that were already present in the pre-pupal gut and which were originally acquired from the mother.

Discussion

Animal symbionts can be passed to offspring through different mechanisms that vary in their reliability of transmission (12). While strict endosymbionts of animals are typically transmitted vertically via eggs, other mechanisms that include an environmental component may also reliably transmit bacteria between generations (15, 20). Here, we examined the mechanisms of bacterial transmission from *Nicrophorus vespilloides* mothers to offspring. *Nicrophorus* larvae are exposed to and consume high densities of bacteria throughout their development on decomposing carrion (26–29, 33). In earlier studies we and others showed that parental care, including preservation of the carcass through secretion of lysozyme, a broad-spectrum antibacterial with greater specificity towards Gram-positive bacteria, and potentially other antimicrobials, is essential for maintaining larval fitness (22, 27, 28, 47). Additionally, preliminary metagenomic analyses from our own lab (unpublished data) and published studies from others (29) have found that parental beetles significantly modify the bacterial composition of decomposing carrion, thereby potentially influencing the bacteria that larvae are exposed to and ingest.

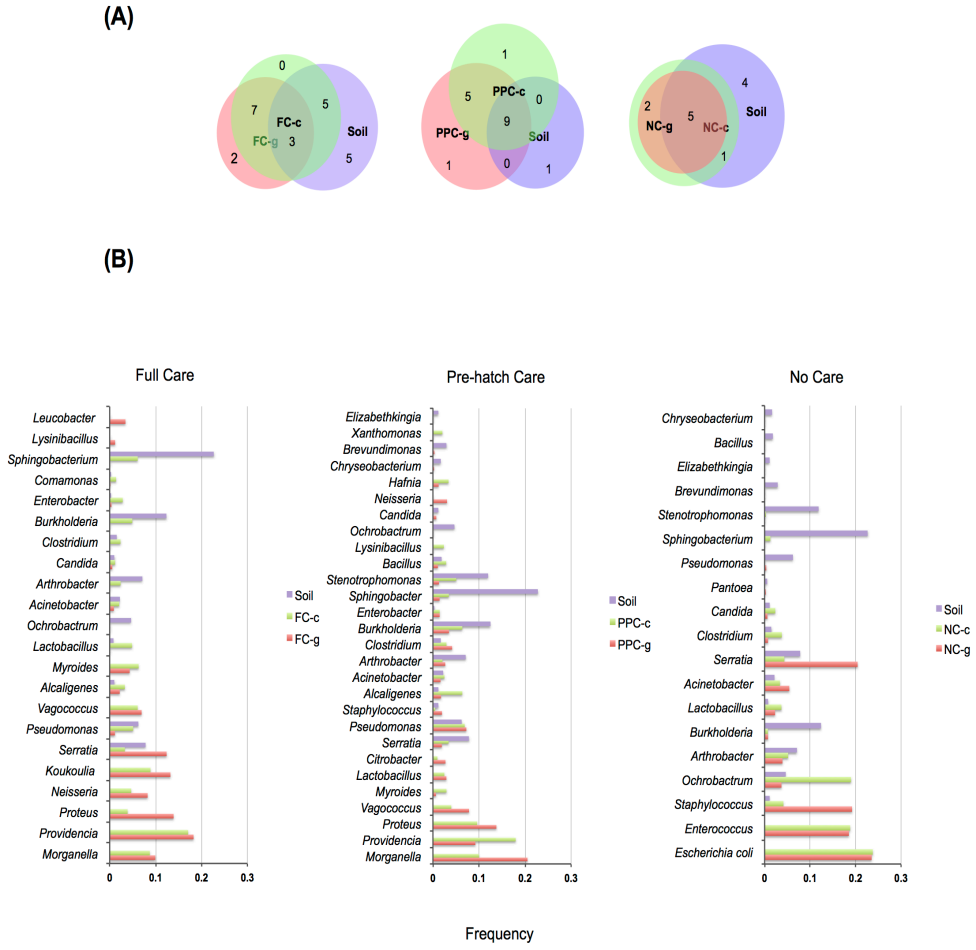

Figure 4. Recolonization of bacterial communities through pupations. (A) Shared and unique genera among treatment groups. Subscripts correspond to the site of isolation, e.g., FC-g corresponds to gut samples, FC-c corresponds to the pupal carapace in the wall of the pupal chamber, and Soil corresponds to bulk soil outside the pupal chamber. (B) Comparison of gut bacterial communities from each sample site and treatment. Strains with frequencies lower than 1% across all communities were excluded from plots.

To examine the influence of parental care on the transmission of bacteria from parents to offspring, we manipulated the level of care parents provide to their larvae. With full parental care, parents apply oral and anal secretions to the carcass both before larvae hatch and throughout larval development (22, 27, 28); they also regurgitate food to larvae during the first three to four days of development (25, 30). As expected, given the continuous direct and indirect exposure to parental bacteria, larvae in this treatment were colonized predominantly with parental symbionts (Fig 2); importantly, despite limitations associated with a CFU-based approach, we observed broad overlap between the dominant bacterial species we cultured and those identified using sequence-based approaches (e.g *Providencia*, *Morganella*, *Vagococcus*, *Proteus*, *Koukoulia*, and *Serratia*) (33). However, with this Full Care treatment alone, it could not be determined if larvae require constant replenishment of the parental species for these to be maintained in the larval gut, given other research showing that gut bacteria may be transient without continuous parental provisions (24, 48, 49). One possibility, for example, is that the dominant bacteria from the carcass could outcompete endogenous beetle bacteria within the larval gut; this could be driven actively, if the bacteria on the carcass are particularly good colonizers, or passively since larval exposure to carcass bacteria is continuous. To address this question, we established broods that only received pre-hatch care. In this treatment, parents have no direct exposure to larvae, and can only influence larval exposure to bacteria indirectly through their influence on the carcass. It is important to note that because eggs are sterile, transmission is also prevented through this route (50). As with the Full Care treatment, larvae receiving only Pre-hatch care were also predominantly colonized by maternal bacteria (Figs 2 and 3). This was not due to an inability of bacteria from the gut to colonize larvae, as larvae in the No-Care treatment were also colonized by a high-density bacterial microbiota. Also, bacteria in the pre-hatch groups were partially colonized by carcass-derived bacteria (Figs 2 and 3C), leading to higher bacterial diversity overall in this group (Supplemental Table 1) and indicating the capacity for carcass-derived bacteria to establish themselves within the larval gut. Rather, we interpret this result to indicate that "endogenous" bacteria from the mother are able to outcompete the carrion associated microbes. Furthermore, this effect is long-lasting and can persist entirely in the absence of direct maternal feeding. Although this interpretation is consistent with our data, this hypothesis will require experimental testing using the culturable species we have now established in our collection of *N. vespilloides* symbionts.

At present, we understand relatively few of the mechanisms used by parents to manipulate the carcass bacteria. However, several factors are likely to be important. First, when parents locate a carcass they strip it of fur, while simultaneously coating the carcass surface with oral and anal secretions. The composition of these secretions has only been partially characterized, but a key component is lysozyme (22, 27). Additionally, oral secretions contain bacteria that can serve as an inoculum to feeding larvae (unpublished results). In addition to these behaviors, we have also observed parents opening the carcass and removing the mouse gut, behaviors that could potentially have a dramatic influence on larval bacterial exposure by introducing oxygen that could bias the bacterial community towards aerobic species or more simply by directly reducing the overall density of bacteria to which larvae are exposed. Following gut removal, parents continue to coat the carcass in secretions and then bury the balled up carrion underground (25, 51), which could influence moisture or temperature levels. Both behaviors could possibly bias the persisting microbial species, and potentially in favor of species originally introduced by caring parents. In addition, caring parents and their larvae may be exposed to different bacterial numbers and composition as a function of carcass age, a factor that is known to have a dramatic influence on larval fitness (26, 52). Although much remains to be determined of these processes, our results clarify the importance of more completely understanding how parents influence both the bacteria on the carcass and how this, in turn, affects larval microbiota establishment.

After larvae complete feeding, they migrate into the soil to pupate (25, 51). Bacterial numbers during this stage decline precipitously (Fig. 1), in part due to the absence of feeding and also to the evacuation of the larval gut. In addition, larvae in some metamorphosing insects undergo a pre-pupal molt which would further reduce bacterial numbers (45, 53). Regardless of the mechanisms, our data are consistent with *Nicrophorus* larvae become effectively sterile during pupation, an outcome previously seen in several flies and mosquitoes (54–56). It is possible that host immunity facilitates pupal symbiont suppression during metamorphosis (53, 57, 58), as a decline of phagocytic haemocytes and an increasing phenoloxidase activity were both detected in *Nicrophorus* pupa (59). Following this aposymbiotic state, bacterial densities are quickly recovered at eclosion with bacterial communities that significantly overlap with those present prior to pupation (Figs 1 and 2). To determine the source of recolonization, we sampled bacteria from the pupal molt as well as the wall of the pupal chambers, and in both cases we observed striking similarity to

the microbial communities of earlier developmental stages. Interestingly, this was true for all treatment groups, suggesting that there is no intrinsic bias to recolonization, but rather that eclosing beetles are colonized by a subset of the bacterial species present in the pupal chamber.

The larval gut of *N. vespilloides* thus appears to be colonized via a combination of mechanisms that are dependent on the degree of parental care and the stage of development. With complete parental care, parents transmit bacteria to larvae through a combination of direct feeding and through an indirect effect mediated by the carcass. At present, it remains unclear if this latter component is because *Nicrophorus* symbionts outcompete the mouse carrion microbiota within the larval gut, or if this occurs primarily on the carcass surface itself. However, the former seems more likely given the vast differences in larval exposure to these two groups of bacteria, and the fact that larvae in the pre-hatch group remained colonized by beetle symbionts, despite lacking any direct exposure to parents (Fig. 2). It is tempting, given the reliable mode of transmission from parents to larvae, to speculate about the function of these symbionts for *Nicrophorus* growth and development, particularly the role of these bacteria in limiting infection from carrion-borne bacteria (29, 33). It will also be important to supplement our studies using laboratory populations of *Nicrophorus* with work focusing on field derived beetle adults and larvae, as exposure to the broader diversity of natural bacteria in the soil or carcass could potentially influence bacterial acquisition and transmission through distinct developmental stages (33). However, this remains an active area of research that we will hope to address in future publications. In addition, it will be important to supplement the present work with more detailed analyses based upon sequencing (29, 33). Although culture-based methods play an essential role in unraveling the relationships between invertebrate host sociality and their symbiont strain-level diversity (60), they are clearly complementary to sequence-based methods that can recover bacterial groups that may be difficult or impossible to culture in the laboratory. Our work clarifies the key links between *Nicrophorus* social behavior and symbiont transmission. This is likely to have parallels in other animal systems where parents invest in the care of offspring.

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Supplementary information

Table S1: Diversity indexes for gut bacterial communities through development.

Group	Richness	Shannon-Wiener Index	Evenness
Full Care	14 ±3	1.959 ±0.277	0.814 ±0.115
Pre-hatch Care	23 ±5	2.465 ±0.339	0.881 ±0.121
No Care	8 ±8	1.323 ±0.232	0.772 ±0.135

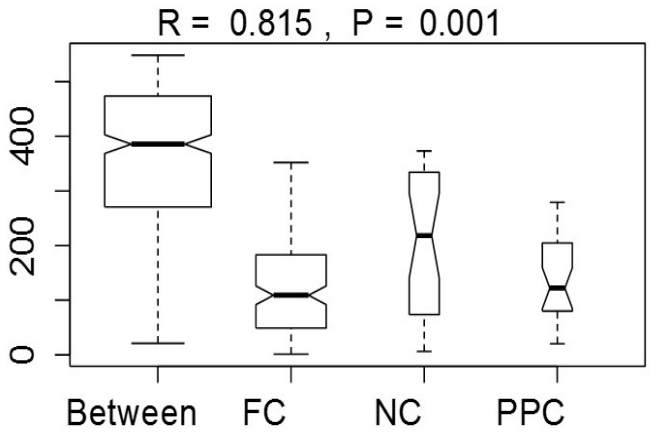

Figure S1 ANOSIM Analysis on Bray-curtis dissimilarity between and within groups of gut bacterial communities. FC, full care beetles; PPC, pre-parental care beetles; NC, no parental care beetles.

Table S2 Mean and SEM (n = 5) of Live counts and CFU. Paired differences were analyzed with paired t-tests.

Day	Mean CFU	SEM	Live cell counts	SEM	% difference	SEM	t	p-value
1	3.95E+03	6.69E+02	4.22E+05	2.30E+05	1.20	0.30	-4.066	0.015
3	7.36E+07	1.57E+07	2.00E+07	7.07E+06	442.35	132.12	5.562	0.005
6	2.73E+06	1.25E+06	1.16E+07	7.87E+06	43.51	24.31	-2.361	0.078
9	7.42E+04	9.56E+04	4.22E+05	3.55E+05	17.18	3.97	-2.847	0.047
12	1.30E+04	8.40E+03	4.60E+04	5.32E+04	46.88	14.49	-1.437	0.224
15	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00	0.00		
18	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00	0.00		
21	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00	0.00		
24	2.46E+04	2.61E+04	6.40E+05	2.41E+05	4.58	2.18	-5.839	0.004
27	1.20E+04	7.59E+03	2.18E+05	2.67E+05	36.79	19.05	-1.684	0.167
30	8.37E+03	7.18E+03	2.10E+04	6.52E+03	56.61	23.61	-2.052	0.109

Chapter 4

Gut microbiota in the burying beetle, *Nicrophorus vespilloides*, provide colonization resistance against larval bacterial pathogens

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Abstract

Carrion beetles, *Nicrophorus vespilloides*, are reared on decomposing carrion where larvae are exposed to high populations of carcass-derived bacteria. Larvae do not become colonized with these bacteria but instead are colonized with the gut microbiome of their parents, suggesting that bacteria in the beetle microbiome outcompete the carcass-derived species for larval colonization. Here we test this hypothesis and quantify the fitness consequences of colonization with different bacterial symbionts. First, we show that beetles colonized by their endogenous microbiome produce heavier broods than those colonized with carcass-bacteria. Next, we show that bacteria from the endogenous microbiome, including *Providencia rettgeri* and *Morganella morganii*, are better colonizers of the beetle gut and can outcompete non-endogenous species, including *Serratia marcescens* and *Escherichia coli*, during in vivo competition. Finally, we find that *Providencia* and *Morganella* provide beetles with colonization resistance against *Serratia* and thereby reduce *Serratia*-induced larval mortality. This effect is eliminated in larvae first colonized by *Serratia*, suggesting that while competition within the larval gut is determined by priority effects, these effects are less important for *Serratia*-induced mortality. Our work suggest that an unappreciated benefit of parental care in *N.vespilloides* is the social transmission of the microbiome from parents to offspring.

Key words: Microbiome, Bacterial symbionts, Colonization resistance, *Nicrophorus vespilloides*

Introduction

Animals are colonized by a diverse array of bacterial symbionts, the microbiome, that provide essential functions to their hosts (1-3). Animal microbiomes can alter nutrient uptake (4), development (5), parasite susceptibility (6), and even behaviors like mate choice (7). In addition, symbionts that reside within animal guts can provide their hosts with resistance to bacterial pathogens via a process called colonization resistance (8). For example, the gut bacterial community of locusts, *Schistocerca gregaria*, prevents invasion and disease from the insect pathogen *Serratia marcescens*, an outcome that depends in part on the diversity of the gut microbial community (9). Similarly, honeybees became more susceptible to *Serratia* infection following treatment with antibiotics that altered the structure of their endogenous microbiota (10). These results support the idea that gut bacteria can provide protection against pathogens while also highlighting the importance and timing of symbiont transmission in juvenile animals (11). However, it is often unclear if colonization resistance results from specific inhibition of invading pathogens or whether it results from the simple fact that symbionts get there first (9, 12, 13). In other words, is colonization resistance the result of specificity or priority?

To address this question we focus on the role of the endogenous microbiota of the burying beetle, *Nicrophorus vespilloides*. This system is especially well suited to this work given the high exposure of larval beetles to environmental bacteria (14-18), together with extensive data on the composition and transmission of the beetle microbiome from parents to offspring (18-22). *N. vespilloides* larvae are reared on small vertebrate carcasses where they feed directly from the carcass and are provided regurgitated food from parent beetles that care for developing broods (14, 23, 24). Parental beetles dramatically increase larval growth and fitness during brood rearing by investing in pre- and post-hatch care (15, 24-26). During pre-hatch care, parents remove the fur and guts of the carcass and coat its surface in oral and anal secretions that have antimicrobial activity (4, 27-29). Post-hatch, parents defend their developing larvae from other insect species and also feed larvae with regurgitated food (23, 30). In a recent study we found that parents transmit their gut microbiome to their larvae by direct feeding. In addition, we found that the core members

of this microbiota could even be transmitted to larvae indirectly, by bacteria deposited onto the carcass by parents (20). This unexpected result suggested that these core bacterial species were outcompeting the numerous microbes living on and inside the carcass within the larval gut, thus giving rise to the stable endogenous *Nicrophorus* microbiome (20, 21). However, the mechanisms of their increased competitiveness remained unclear as were the consequences of their colonization.

Here we carry out invasion experiments into sterile larvae to directly quantify competitive interactions taking place between endogenous and non-endogenous microbes from the *Nicrophorus* gut. We first quantify bacterial growth rates within the larval gut and then directly determine the competitive interactions between species during mixed inoculations and in different orders. Finally, we quantify whether members of the core microbiome provide colonization resistance against *Serratia marcescens*, a known insect pathogen (31-33). Briefly, we show that native gut species significantly outcompete foreign species within the host gut, irrespective of infection order. In addition, we find that the endogenous microbiota increases beetle fitness, both in terms of brood size and in terms of pathogen resistance in larvae. Our results provide strong evidence that an important benefit of parental care in *N. vespilloides* is the social transmission of the microbiome from caring parents to their offspring.

Methods and Materials

Beetle collection and rearing

Experimental beetles were taken from an outbred laboratory population derived from wild-caught *N. vespilloides* individuals trapped near Leiden in The Netherlands, between May and June 2015. Beetles were maintained in the laboratory at 20°C with a 15:9 hour light:dark cycle and fed fresh chicken liver twice a week. Mating pairs were established by placing a male and female in a small plastic container containing ~ 1 cm soil overnight. Mated females were provided with a fresh carcass (20-23g) the following morning to initiate egg laying.

To examine the impact of different microbial communities on *N.*

vespilloides fitness, we established independent treatment populations containing endogenous or carcass derived gut bacteria, designated FC (full-care) and NC (no-care) beetles respectively. Whereas parents and larvae in the FC treatment were reared in the presence of parental care and thus acquired their microbiota primarily from their parents, larvae in the NC group were reared in the absence of parental care (with an unprepared carcass that we opened using a sterile scalpel), and acquired their microbiota from the carcass and surrounding soil (20). Ten day old adults that had eclosed from FC and NC broods were paired within treatments for mating (n = 15 / treatment) and subsequently provided with a fresh mouse carcass (22-24g) for breeding. To control for the possible effects of female size on reproductive output (Steiger 2013), females from each treatment were size matched prior to establishing their own broods (mean +/- SD: NC: 0.02 +/- 0.198, FC: 0.25 +/- 0.026; t25 = 0.759, p = 0.455). The fitness of both parental treatment groups (NC and FC) was determined by quantifying total brood size, total larval weight and mean larval mass.

Experimental bacterial inoculation of *N. vespilloides* larvae

To generate germ-free larvae, we collected eggs 15 hours after FC females were provided with a fresh carcass. These were surface sterilized twice for 15 minutes in an antimicrobial solution containing hen-egg white lysozyme (1 mg/ml), streptomycin (500 µg/ml) and ampicillin (100 µg/ml), and followed by a sterile water wash. Next, treated eggs were transferred onto 1% water agar plates to hatch. Previous experiments have shown that eggs thus treated are free of bacteria (Jacobs et al. 2014). 0 - 24h old first-instar larvae were transferred onto new sterile 1% water agar petri dishes (100mm x 15mm) in groups of a maximum of 7 larvae. Larvae on each plate were derived from independent breeding pairs. Larvae were fed a sterile diet developed using Pasteurized chicken liver prepared via a "Sous vide" cooking approach. Fresh chicken liver was sliced into 3g chunks using aseptic technique and transferred in individual pieces to a 1.5ml eppendorf tube containing 100 µl sterile water. These were then placed in a water bath at 65°C for 8 minutes, followed by immediate cooling at -20°C. We determined the effectiveness of this method by plating liver samples before and after pasteurization onto both 1/3 strength Tryptic Soy agar and LB agar. The initial CFU of unpasteurized liver was ~ 1e6/gram CFU while following treatment the CFU was reduced to 0 (with a limit of detection of ~ 10 CFU/mL). Larvae were offered this sterile diet, alone

or coated with different bacterial inocula, on new 1% water agar plates daily.

In vivo competition within larvae

To determine if "endogenous" bacteria can outcompete foreign strains during larval colonization we competed bacterial strains against one another within the larval gut, focusing on four different bacterial species. The bacterial species *Providencia rettgeri* and *Morganella morganii* are abundant *N. vespilloides* gut symbionts throughout development and are considered "endogenous" species (Wang & Rozen 2017; Vogel et al. 2017). By contrast, *Serratia marcescens* and *Escherichia coli*, which are found commonly in both soil and on decomposing carcasses, colonize larvae that are reared without parental care in NC broods (20). *S. marcescens* is also a known insect pathogen in several insect species (31-33), including *N. vespilloides*. *P. rettgeri* (P) and *M. morganii* (M) were isolated from *N. vespilloides* adults guts while *S. marcescens* (S) and *E. coli* (E) were isolated from decomposing mouse carcasses (20).

Bacteria for inoculations were cultured overnight at 30°C in 1/3 TSB medium. Overnight cultures of each species were pelleted and washed two times in sterile phosphate buffered saline (PBS, pH= 7.2), and diluted to an optical density at 600nm (OD600) of 0.2 measured using a BIO-RAD SmartSpecTM Plus spectrophotometer. Ten microliters of this solution, containing ~10^6 cells total, was used to coat sterile liver prepared as above. Inoculations with 2 species contained the same total bacterial density, with each species present at a 1:1 ratio. Larvae were provided with inoculated diet for six hours on a sterile water agar plate, after which they were transferred to a new agar plate containing new sterile diet. Subsequent transfers to plates containing fresh sterile food took place every 24 hours for 7 days, or until larvae were destructively sampled. In experiments where larvae were sequentially challenged with different bacterial species, we treated larvae the same as above, but larvae were inoculated with target strains in series: the first as above, and the second 6 hours later on a new plate containing diet coated with the second bacterial strain. As with the first exposure, larvae were exposed to bacteria in the second inoculum for 6 hours, after which they were returned to a sterile plate with sterile diet.

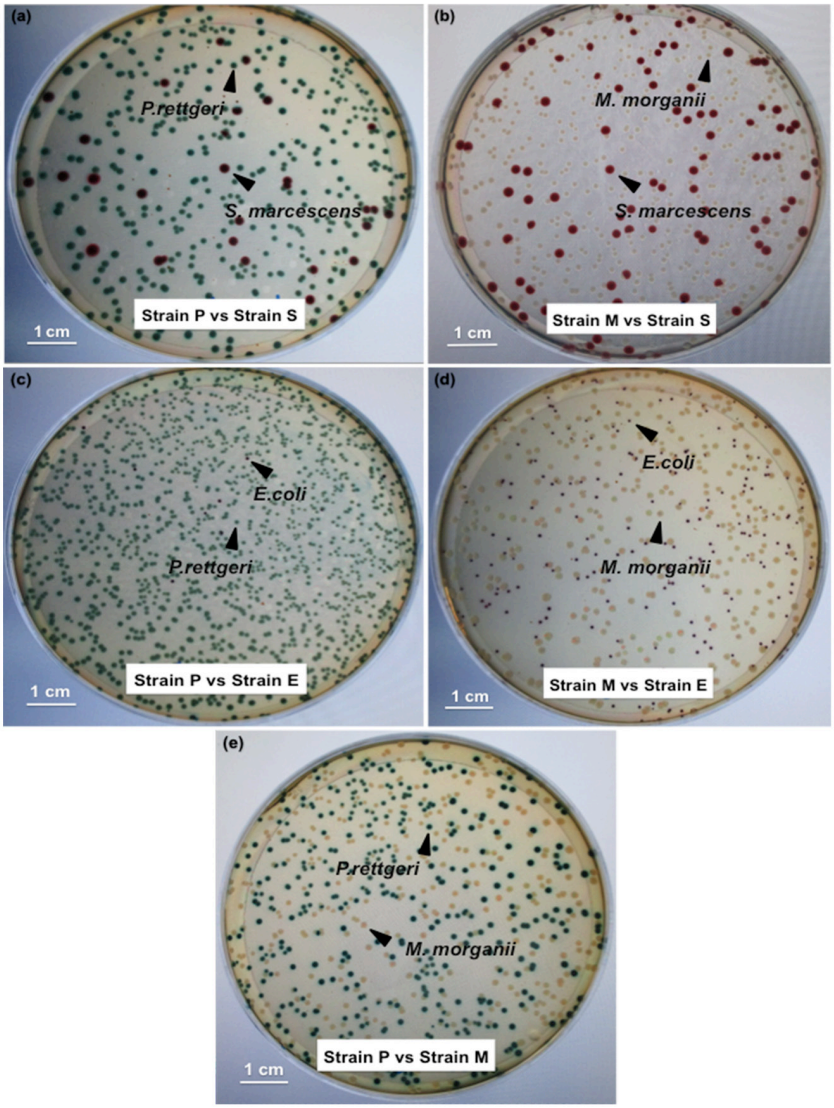


Figure 1. Color and morphology of experimental strains on chromogenic agar plates (CHROMagarTM Orientation) used for bacterial competition assays. Bacterial combinations shown are: P vs S (a); M vs S (b); P vs E (c); M vs E (d); P and M (e).

To examine competitive interactions within the *Nicrophorus* gut, larvae were inoculated either simultaneously or in series with two of the four species in the following pairings (Strain 1 vs Strain 2): P vs S; M vs S; P vs E; M vs E; P vs M; and S vs E. Within each treatment, larvae from independent families

(n = 6) were inoculated as outlined above and then 6 larvae were destructively sampled for plating 6h or 24h later. These values were taken as estimates of input and final densities, respectively. Competition indices (CI) were calculated using the following equation: CI = (Strain 1 output /Strain 2 output) / (Strain 1 input / Strain 2 input), where input and output values refer to initial and final densities of each competitor, respectively. CI w log transformed, so that a CI of 0 indicates equal competitiveness, while CI > 0 indicates that the strain 1 is a stronger in vivo competitor. A two-tailed t-test was used to test if CI values for each strain differed significantly from 0.

At each time point, larvae were sampled by sterilely dissecting individual larval guts with fine forceps and suspending these in 0.7 ml sterile PBS. Gut contents were serially diluted in PBS and plated to quantify CFU on a chromogenic medium (CHROMagarTM Orientation), which can distinguish our experimental strains based on both color and morphology (Figure 1). Mortality rates for each treatment were: P vs S (38.1%); M vs S (47.6%) and P vs M (35%), P vs E (54.5%); M vs E (64.7%) and S vs E (71.4%).

Larval fitness with different bacterial colonizers

To determine the impact of different bacterial symbionts on larval survival, larvae were inoculated as above and then monitored for survival through time. Larvae exposed to sterile PBS (pH = 7.2) were used as a control in this experiment. A minimum of 40 larvae from 9 - 15 families was collected for each bacterial treatment. We monitored larval survival every 24 hours after inoculation. To reduce the high rates of mortality in larvae reared on liver, all the experimental larvae were transferred daily into a fresh petri dish containing fresh sterile diet.

Statistical Analysis

Parental fitness and bacterial colonization data were analyzed using ANOVA. Larval survival data was analyzed by fitting a Cox proportional hazard model; this model was constructed by fitting a saturated model using treatment, block and treatment*brood interactions as covariates. The Wald's test was used to compare mortality between treatments. All analyses were conducted using SPSS version 24 (IBM SPSS Inc., Chicago, IL, U.S.A.).

Results

The effects of gut microbiota on parental fitness

To examine the role of the parental gut microbiome on beetle fitness, we reared larvae either with (FC) or without parental care (NC) and then mated the dispersed adults within treatments and allowed them to rear broods on fresh carcasses. Through this treatment, all parents were given the opportunity to rear offspring under identical conditions, and there were neither differences in carcass or maternal weight (both NS). Previous results have shown that parents from these different rearing conditions differ significantly in microbiome composition (20), the FC individuals containing an endogenous symbiont population and the NC individuals a microbial population derived from the soil and the decomposing carcass. Our results show that gut microbiomes have a significant influence on parental fitness (Figure 2). FC parents produced significantly heavier broods than NC parents, irrespective of brood size (2-tailed ANCOVA: $F_{1,27} = 6.09$, $p = 0.021$).

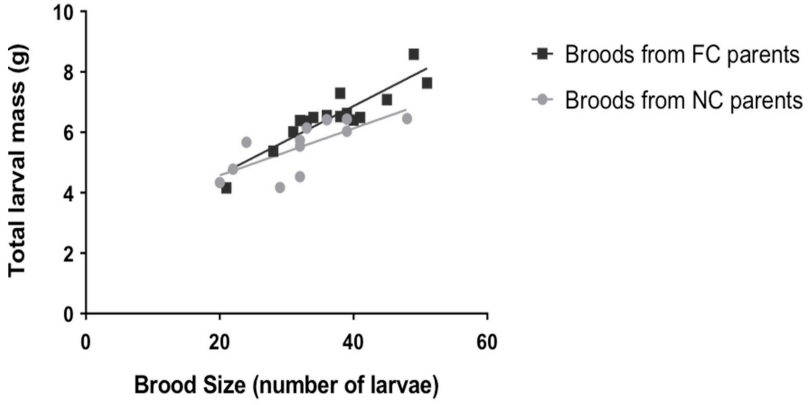


Figure 2. Total larval mass as a function of brood size for maternal beetles that were reared with either Full Care (FC) or No Care (NC).

Competitive interactions in vivo

To study competitive interactions between bacteria during larval colonization, we selected four focal species to examine in detail. Two species,

Providencia rettgeri and *Morganella morganii* are common endogenous colonizers of the beetle gut (18, 20, 21), while the other two, *S. marcescens* and *E. coli*, are found more commonly in the guts of beetles reared without parental care (20). We first inoculated beetles with each species alone to measure growth and colonization. Figure 3 shows that while all four species are able to colonize the larval gut, their ability to increase in density in vivo varies significantly between strains (2-tailed ANOVA: $F_{3, 12} = 43.13$, $p < 0.001$).

Competitive interactions between species were next determined using in vivo pairwise assays where two species were simultaneously inoculated into 1 day old larvae. Consistent with expectations based on mono-associated larvae, we observed clear competitive differences between the strains. *Providencia* and *Morganella* significantly outcompeted both *Serratia* (P vs S: $t_5 = 2.52$, $p = 0.053$; M vs S: $t_3 = 4.42$, $p = 0.022$) and *E. coli* (P vs E: $t_2 = 11.26$, $p < 0.001$; M vs E: $t_3 = 5.89$, $p = 0.01$), although to different degrees. By contrast, there were no significant competitive differences between *Providencia* and *Morganella* (P vs M: $t_4 = 2.16$, $p = 0.097$) (Figure 4A).

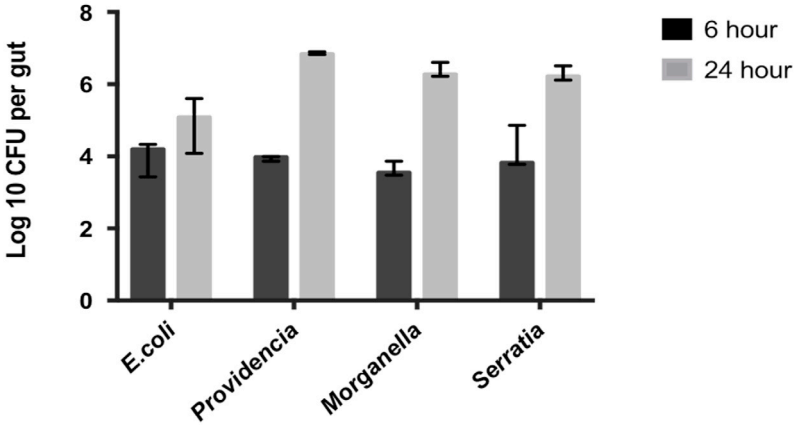


Figure 3. Growth and colonization of *Nicrophorus vespilloides* symbionts within the larval gut over 24 hr. Values correspond to the mean ±95% CI.

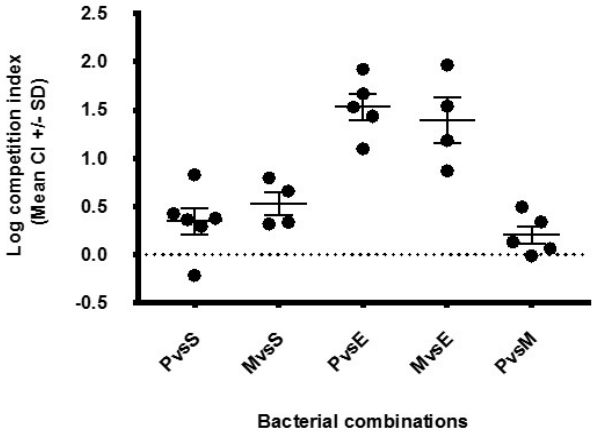
We next determined if competitive interactions between *Providencia* and *Serratia* were influenced by the order of inoculation. Specifically, we were interested in determining if the outcome of competition was reversed in larvae that were first inoculated with *Serratia*. Our results in Figure 4B clarify that order is not an important determinant of competitive fitness (2-tailed

ANOVA: $F_{2, 29} = 0.59$, $p = 0.56$). *Providencia* outcompetes *Serratia* in all cases to a similar degree regardless of the order of inoculation.

Larval survival with different bacterial colonizers

Our results show that the endogenous microbiome provides likely benefits to *Nicrophorus* by increasing total brood mass, and also that key members of this microbiome can outcompete species that are predominantly found in larvae that do not receive parental care. To test if these competitive interactions translate into differences in larval fitness, we measured the survival of larvae inoculated with single or multiple strains, as above. Results in Figure 5A show that larval mortality varies significantly as a function of their bacterial colonists ($\chi^2 = 11.364$, df = 3, $P < 0.01$), with increased mortality in larvae inoculated with *Serratia* compared to either *Morganella*, *Providencia* or a PBS control (Wald statistic = 6.274; 4.794; 9.202, respectively, all $P < 0.05$). This result is consistent with the known pathogenic effects of *Serratia*. By contrast, there were no significant differences in mortality between larvae inoculated with either *Providencia* or *Morganella* and the PBS control ($\chi^2 = 0.156$, df = 2, $P = 0.925$).

(A)



(B)

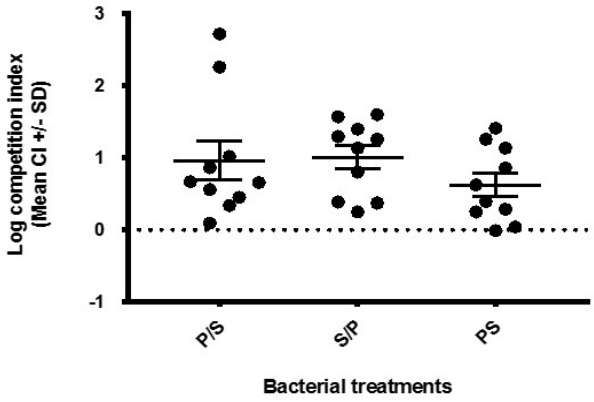


Figure 4. Competitive differences between different bacterial species in vivo within the larval gut. Competition indices (CI) are given in reference to the first species listed on the x-axis for (A) and with respect to *Providencia* for (B). Strains were either inoculated simultaneously (A) or in series (B) in cases where strains are separated with a/(e.g., P/S: *Providencia* was inoculated first and then followed with *Serratia*, whereas in PS both strains were coinoculated). The dashed black line illustrates a CI of 0, which indicates equal competitiveness of two strains. Values >0 indicate that strain 1 is a stronger in vivo competitor.

We also observed significant differences in survival when larvae were simultaneously inoculated with *Serratia* and either of the endogenous species compared to survival when *Serratia* is inoculated alone (χ^2 = 38.767, df = 4, P < 0.001). Most importantly, we found that co-inoculating *Serratia* with *Providencia* and/or *Morganella* significantly increased larval survival, suggesting that these species provide protection via colonization resistance for larvae (Wald statistic of PS; MS; PMS = 8.188; 3.697; 5.102, respectively, all P < 0.05, Figure 5B). However, this benefit of colonization resistance disappeared when *Serratia* was able to become established prior to inoculation with *Providencia*; there were no survival differences between larvae inoculated with *Serratia* twice in series and larvae first inoculated with *Serratia* and then followed by *Providencia* (Wald statistic of S/P = 0.077, P = 0.782, Figure 5C).

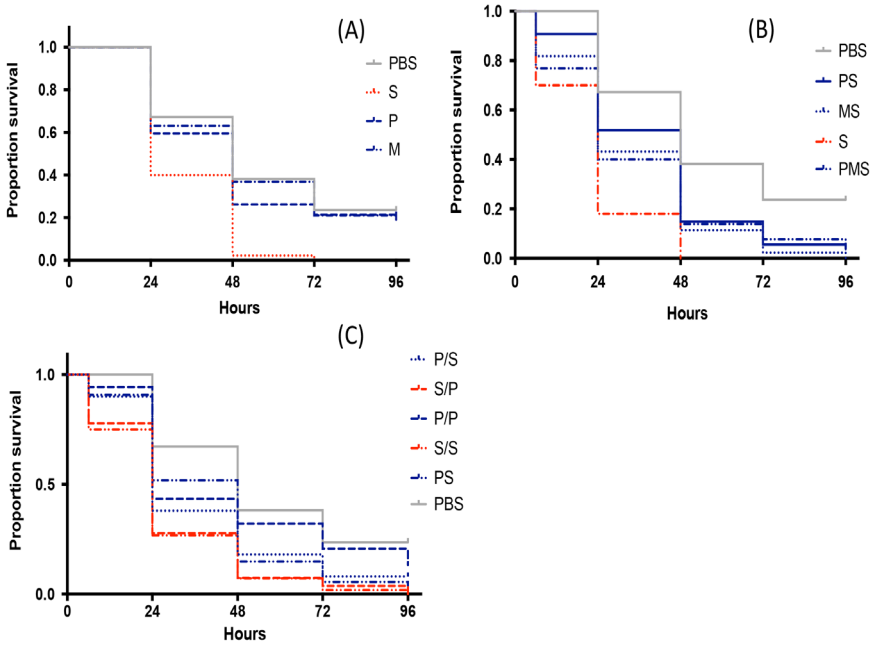


Figure 5. Larval survival when inoculated with different bacterial species. Larvae were inoculated with (A) single bacterial species in monoculture, (B) >1 species in coculture simultaneously, or (C) bacteria either simultaneously or in series. Bacteria inoculated simultaneously are designated with the first letter of the species name (e.g., PS = *Providencia* with *Serratia*), while species inoculated in series are given in the same way with a slash (e.g., P/S = *Providencia* followed by *Serratia*). A PBS (phosphate-buffered saline) control was set up for all the experiments.

In light of results in Figure 4B showing that order of inoculation does not affect *Providencia* competitiveness, these survival results indicate that *Serratia* induced larval mortality is insensitive to bacterial competitiveness, thus supporting the idea that initial establishment of the endogenous microbiota is crucial for colonization resistance.

Discussion

Nicrophorus larvae are exposed to a highly diverse microbiota in their

breeding environment, first from the soil where they hatch and next from the microbes proliferating on and within their carrion resource. In the absence of parental care, larvae become colonized with these bacteria (20) which reduces their weight and survival (15), and also leads to reduced brood mass when these larvae reproduce as parents (Figure 2). However, when larvae are reared with parental care, their gut microbiome resembles that of their parent, even if parental care is limited to carcass preparation prior to larval hatch (20). These results suggested that the bacteria within the parental gut are better competitors for the larval gut, but our earlier work neither tested the colonization potential and competitiveness of the constituent species nor determined the consequences of colonization with the *Nicrophorus* "endogenous" microbiome. Our aims here were therefore to address these questions experimentally by inoculating different endogenous or non-endogenous bacterial species into the guts of developing larvae. We focus specifically on four species: *Providencia rettgeri* and *Morganella morganii*, that are dominant members of the larval microbiome (20, 21), and *Escherichia coli* and *Serratia marcescens*, which are non-endogenous species, but which are either observed in the larval gut (*Serratia*) or have the potential to colonize it through exposure on the mouse carcass (*E. coli*) (20, 36).

Using this approach, we first determined that there are clear differences in the colonization potential of different bacterial species. While *Providencia*, *Morganella* and *Serratia* increase in density more than 100-fold in 24 hours within the larval gut, *E. coli* was a poor colonizer and only increased by ~ 10-fold over the same time interval (Figure 3). In addition to clarifying these differences, these experiments also established that it is feasible to experimentally colonize larval beetles via diet manipulation. The growth differences between strains in monoculture were reflected in their interactions in vivo during co-culture. Specifically, we saw competitive dominance of *Providencia* and *Morganella* over *E. coli* and *Serratia* when pairs of strains were simultaneously fed to larvae (Figure 4A). Moreover, in competition experiments between *Providencia* and *Serratia*, we found that the order of inoculation did not affect the competitive outcome between strains (Figure 4B). This latter result suggested that priority effects are not realized in this system because *Serratia* could be displaced even after a 24-hr head start in colonization. By contrast, another recent study found that the colonization competitiveness of *Borrelia* strains within the mouse gut are significantly determined by their order of presentation to the host mouse (37).

At present, we have limited understanding of the factors that mediate the competitive differences between strains within the *Nicrophorus* larval gut. Differences in in vivo growth rates are sufficient to explain the competition results during simultaneous inoculation. However, the fact that *Providencia* can still invade an established *Serratia*-colonized larva (Figure 4B), suggests the possibility that competitive interactions are in part mediated by the host. For instance, host innate immunity could be a direct factor in determining the competitive outcome and final population density of bacterial species within hosts (38). Equally, commensal bacteria could prime the host immune response to limit pathogen colonization by causing an up-regulation of antimicrobial peptides, such as AMP molecules in *Aedes aegypti* mosquitoes and islet-derived protein 3γ in mice (39, 40); however, these would need to be specifically targeted to non-symbiont species. Host involvement in this system is further suggested by other experimental results showing that in vitro, *Serratia* is able to outcompete *Providencia* (YW unpublished data), a result likely attributed to the faster growth of this strain during in vitro culture. An important aim for future work will be to clarify the factors that drive the competitive interactions between bacterial strains during colonization.

To understand the consequences of the *Nicrophorus* microbiome for beetle fitness, we quantified larval mortality following inoculation of monocultures or co-cultures of different bacterial species. Consistent with results on colonization resistance in other systems (9, 41), these experiments showed that *P. rettgeri* and *M. morganii* both provide protection against *Serratia* infection, but with no added protection if both endogenous species are present (Figure 5B). This is expected given the results from in vivo competition assays. By contrast, when *Serratia* is inoculated first, the protection provided by *Providencia* is abolished, in spite of the fact that *Providencia* can outcompete *Serratia* in these conditions (Figure 5C). Interestingly, these results indicate that the pathogenesis of *Serratia* is separate from its in vivo competitive ability, perhaps owing to toxin production or invasion through the gut into the haemocoel within the first 24 hours (42-44). Thus initial establishment of the endogenous microbiota is apparently crucial for colonization resistance.

Although the mechanism of *Serratia*-induced mortality remain unknown in this species, the fact that colonization resistance requires the prior or simultaneous establishment of the *Nicrophorus* endogenous microbiota has important implications for our understanding of the functions of parental

care. Parents protect larvae and provide nutrition in the form of regurgitated food (24). In addition, they transfer their gut microbiome to larvae by direct feeding and via contamination of the carcass surface (18, 20, 22). The present results indicate that larvae benefit directly from the acquisition of these bacteria (Figures 2 and 5B), and suggest that two of the key members of the *N. vespilloides* microbiome are mutualists. Thus while *Nicrophorus* adults ensure transmission of these and other species (18, 21) from generation to generation, the bacteria provide direct benefits to beetles within the highly contaminated carcass environment (21). It remains possible that other advantages exist, for example improved nutrient acquisition (46) or changes in the composition of the decomposer microbial community on the carcass (18), but as yet these possibilities have not been measured. In addition, it is important to note that parents may transmit other factors, besides microbes, to offspring during development that may directly or indirectly affect their parental abilities. Larval size tends to be reduced in the absence of parental care and smaller larvae give rise to smaller adults that are less competent parents (34). Although we excluded this confounding factor by using only size-matched female adults (see Methods) it was not possible to fully exclude the potential influence of other transmitted factors, e.g. those affecting immunity or social development, which in turn altered parental care (see (47) for a clear example of such transgenerational effects in earwigs). Using direct microbial inoculations may therefore be worthwhile in future studies.

Our results provide strong evidence that members of the *Nicrophorus* microbiome provide direct advantages to larvae and adults; however, it is important to note that these advantages were not measured in the natural context of the carcass itself. While this was necessary for the current work, it does mean that we may be underestimating larval exposure to potential bacterial pathogens (48). In addition, our assay clearly suffers from extremely high rates of larval mortality, irrespective of treatment. Although the approach we used was essential to avoid reinfection of otherwise sterile larvae, it is possible that the heat treatment in our Sous Vide method rendered the liver diet less nutritious, or possibly modified the competitive environment of the larval gut. Artificial diets, or more ideally germ-free mice as presented by Prof. Rebecca Kilner at the recent meeting for the European Society for Evolutionary Biology, that better mimic the larval environment and that improve larval nutrition and survival are thus needed to more fully elucidate the functions of the *Nicrophorus* microbiome. However, despite these limitations, our results point towards yet another role of parental care in *N. vespilloides*, and argue for

further comparative studies in other congeners that vary in their requirements for parental care.

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Author contributions

Both authors were involved in experimental design and analysis and co-drafted the manuscript. YW conducted all experimental work. Both authors approve of this final submitted manuscript.

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Chapter 5

Fitness costs of phoretic nematodes in the burying beetle, *Nicrophorus vespilloides*

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Abstract

Nicrophorus vespilloides is a social beetle that rears its offspring on decomposing carrion.Wild beetles are frequently associated with two types of macrobial symbionts, mites, and nematodes. Although these organisms are believed to be phoretic commensals that harmlessly use beetles as a means of transfer between carcasses, the role of these symbionts on *N. vespilloides* fitness is poorly understood. Here, we show that nematodes have significant negative effects on beetle fitness across a range of worm densities and also quantify the density-dependent transmission of worms between mating individuals and from parents to offspring. Using field-caught beetles, we provide the first report of a new nematode symbiont in *N. vespilloides*, most closely related to *Rhabditoides regina*, and show that worm densities are highly variable across individuals isolated from nature but do not differ between males and females. Next, by inoculating mating females with increasing densities of nematodes, we show that worm infections significantly reduce brood size, larval survival, and larval mass, and also eliminate the trade-off between brood size and larval mass. Finally, we show that nematodes are efficiently transmitted between mating individuals and from mothers to larvae, directly and indirectly via the carcass, and that worms persist through pupation. These results show that the phoretic nematode *R. regina* can be highly parasitic to burying beetles but can nevertheless persist because of efficient mechanisms of intersexual and intergenerational transmission. Phoretic species are exceptionally common and may cause significant harm to their hosts, even though they rely on these larger species for transmission to new resources. However, this harm may be inevitable and unavoidable if transmission of phoretic symbionts requires nematode proliferation. It will be important to determine the generality of our results for other phoretic associates of animals. It will equally be important to assess the fitness effects of phoretic species under changing resource conditions and in the field where diverse interspecific interactions may exacerbate or reduce the negative effects of phoresy.

Keywords: commensalism, nematode, *Nicrophorus vespilloides*, phoresy

Introduction

Animals that persist on ephemeral and spatially dispersed resources have evolved diverse mechanisms to detect and exploit these resources (1–3). Carrion feeders, like blowflies and burying beetles, can use olfactory cues to detect minute concentrations of the volatile products of animal decomposition and can orient their search flights accordingly (4, 5). However, some animals are incapable of moving across large distances themselves. Instead, these species hitch a ride on the bodies of other more mobile species and are consequently transported from resource to resource (6, 7) Thus rather than developing mechanisms to detect resources, they have evolved mechanisms to ensure reliable and durable associations with the species that carry them (8, 9). This strategy, known as phoresy, is common in many species of insects, mites and nematodes and is a form of symbiosis that is typically believed to be harmless to the host (10, 11). The rationale for this belief is that because phoretic species are wholly dependent on their hosts for their migration, species that cause too much harm and thereby reduce their transport between breeding resources, face the risk of local extinction (11). However, just as parasites and pathogens can evolve levels of virulence that balance harm to hosts with the need to be transmitted between hosts, so too may phoretic species become parasitic, as long as this harm facilitates their transmission between hosts (12–15). Few studies have quantified the direct harm of phoretic species to their hosts while also estimating their persistence and transmission between host individuals and across generations. Our aim in this paper is to address these questions in the context of the burying beetle, *Nicrophorus vespilloides*, and its phoretic nematodes.

Nicrophorus burying beetles are subsocial insects that breed on small vertebrate carrion (16). After locating a small vertebrate carcass using volatile cues produced from microbial decomposition (5), a mated female lays eggs in the surrounding soil after which she (or a mated pair) prepare the carcass for the arrival of the hatched larvae (17). The carcass is buried underground, stripped of fur or feathers, the gut is removed, and then it is coated in antimicrobial oral and anal secretions (18–22). When larvae migrate to the carcass, parents remain to feed them via regurgitation (23, 24), which both provides a meal and also transmits the endogenous microbiome to the developing larvae (25–28). But beetles are not alone in their consumption of the carcass. *Nicrophorus*

adults trapped in the field are conspicuously associated with high densities of mites and nematodes that are attached to their carapace or reside internally (29, 30). Many species of mites have established phoretic associations with burying beetles, and their well-studied effects on beetles range from harmful to beneficial, depending on the context and the study (31–33). By contrast, only one species of phoretic nematode has been described in *Nicrophorus* and its effects on beetles are unknown (30).

Richter (1993) described the carrion-feeding nematode *Rhabditis stammeri* isolated from *N. vespilloides*. He showed that worms were present in the gut and genitalia and could be transmitted between mating individuals (30). However, although *Nicrophorus* researchers regularly comment on the presence of nematodes in laboratory and field populations, there is no direct evidence that these worms are actually *R. stammeri*, nor is there any understanding of their natural abundance in field-caught insects. More importantly, we lack an experimental understanding of the fitness consequences of these nematodes for beetles. As part of our efforts to understand the evolution and ecology of phoretic associates of *N. vespilloides* we provide a detailed study of the identity and effects of a novel nematode associate of *N. vespilloides*, most closely related to *Rhabditoides regina*, that we cultured and quantified from field-caught beetles. In brief, we find that these nematodes are extremely numerous in wild beetles and significantly reduce *N. vespilloides* fitness. In addition, worms are efficiently transmitted in high densities between mating adults and from infected mothers to their offspring, which then persist through beetle development and are retained into adulthood. We discuss these results in the context of the evolution of interspecific interactions in *N.vespilloides* and the evolution of host harm in phoretic species.

Methods

General procedures

All experimental beetles were taken from an outbred laboratory population derived from wild-caught *N. vespilloides* individuals trapped in Warmond, near Leiden in The Netherlands, between May and June 2016. Beetles were maintained in the laboratory at 20°C with a 15:9 hour light:dark cycle. All adults were fed fresh chicken liver twice a week. To maintain the laboratory population and to establish experimental broods, an unrelated male and female

were placed together overnight without food in one small plastic container filled with 1-2 cm of autoclaved soil for mating. The following morning, mated females were provided with a freshly thawed mouse carcass in a new larger container for egg laying. Broods were reared until larvae dispersed from the carcass, approximately 7 days post-hatching (34). Dispersed larvae were placed together into a new container of sterile soil until eclosion, at which point they were removed to new individual containers.

To generate nematode-free adults, eggs were collected from broods within 12-24 hours of laying and surface sterilized with an antimicrobial solution of hen egg white lysozyme (1 mg/ml), streptomycin (500 µg/ml) and ampicillin (100 µg/ml) (18). These were then transferred onto 1% water agar plates to hatch, after which they were placed onto a freshly thawed mouse carcass that had been opened using a sterile scalpel. To prevent nematode transmission from parents to newly hatched larvae, 1st generation nematode-free larvae were reared without parental care. Once these nematode-free individuals had eclosed as adults, they were crossed as above, and maintained thereafter on autoclaved soil.

Nematode quantification from field-caught and lab beetles

Nematodes were collected and counted from the guts and cuticles of field-caught and lab-reared beetles. Individual adult beetles were vortexed for 3 minutes in 700 µl sterile phosphate buffer saline (PBS, pH= 7.2) to collect nematodes from the cuticle. To quantify nematodes from the beetle gut, we removed individual beetle guts with fine forceps and suspended these in 700 µl sterile PBS. 10 µl of each suspension (cuticle or gut sample) was then transferred onto a haemocytometer and examined at 10X magnification for counting. Three independent 10 µl aliquots were counted from each sample to generate a mean estimate/sample.

Nematode maintenance and identification

Experimental nematodes were isolated directly from the cuticles of field-caught beetles. Species identification is explained below. To maintain laboratory populations, newly collected nematodes were transferred onto petri plates containing Nematode Growth Medium (NGM contains: 1.7%

agar/L; 50mM NaCl; 0.25% peptone; 1mM $CaCl_2$; 5 µg/mL Cholesterol; 25mM KH_2PO_4; 1mM $MgSO_4$) (35) and fed with an *E. coli* strain originally isolated from a mouse carcass and held at 20°C. Nematodes were transferred to fresh plates containing *E. coli* at an intial density of ~10^6 cells/plate every 2 days.

To determine species identity, nematode samples reared on NGM plates were collected and suspended in sterile PBS (100mM, pH 7.2), after which they were surface sterilized in a wash solution containing a 1:2 ratio of 5N NaOH and a 5% solution of sodium hypochlorite (35). Washed nematodes were re-suspended in 1ml PBS, and then centrifuged at 13,000 × g for 10 min. Nematode pellets were re-suspended in 0.7 ml PBS and stored at -20 °C. For DNA extraction, nematode samples were thawed and homogenized with a sterile micropestle and vortexed for 2 mins. Samples were then lysed in SDS at 60°C for 30 min. following the method of Donn et al. (36). DNA was extracted using phenol-chloroform and quantified using a Thermo NanoDrop ND-1000 spectrophotometer. The 18S rRNA gene fragment (~ 900 bp) was amplified using primer pairs Nem_18S_F (CGCGAATRGCTCATACAACAGC) and Nem_18S_R (GGGCGGTATCTGATCGCC) (37). For PCR amplification, 2µl of template containing 2-10 ng DNA was used directly in a 20µl reaction mixture using *Pyrococcus furiosus* (Pfu) DNA Polymerase. PCR was performed in a thermal cycler (Bio-RAD T100TM) with thermal cycling of 95 °C for 5 min, followed by 35 cycles of 95 °C for 30 sec, 54 °C for 30 sec, 72 °C for 30 sec and a final extension at 72 °C for 5 min. PCR products (fragment length of ~ 900 bp) were gel purified (illustraTM GFXTM PCR DNA and Gel Band Purification Kit) and sequenced commercially via MaxyGen. The resulting 18s rRNA gene sequence was classified to species using a nucleotide BLAST against the NCBI database.

Fitness effects and transmission of nematode infections

To determine the fitness effects of nematodes on beetles, broods were established with worm-inoculated mated females (at least 20 broods/ inoculation density). All broods were established with virgin females that had eclosed at least 7 days prior to mating. Before inoculation, nematodes were surface sterilized to remove any surface-associated bacteria, and then suspended in sterile PBS. Worm densities were quantified prior to inoculation by direct counts using a haemocytometer. Experimental worm-free beetles were inoculated with either ~10, 102, 103 and 104 nematodes per beetle by

Table 1: Summary statistics for broods produced by worm-free females and females infected with different densities of nematodes prior to mating.

Infection level	Number of broods	Brood size (excluding failed broods)	Total brood mass	Mean larval mass	Number of eclosed adults	Fraction eclosed
0	20	27.25 +/- 1.78	5.67 +/- 0.21	0.181 +/- 0.006	28.82 +/- 1.46	0.91 +/- 0.03
10	32	17.41 +/- 2.07	3.03 +/- 0.29	0.165 +/- 0.005	12.85 +/- 1.67	0.68 +/- 0.07
100	32	19.90 +/- 2.22	3.65 +/- 0.36	0.171 +/- 0.004	13.81 +/- 1.84	0.59 +/- 0.05
1000	32	13.41 +/- 1.88	2.62 +/- 0.28	0.162 +/- 0.005	5.88 +/- 1.54	0.36 +/- 0.07
10000	32	11.21 +/- 2.15	2.34 +/- 0.36	0.156 +/- 0.007	10.45 +/- 2.01	0.61 +/- 0.07
ANCOVA		$p < 0.001$ $F_{4,112} = 8.49$	$p < 0.001$ $F_{4,112} = 14.48$	$p = 0.016$ $F_{4,112} = 3.2$	$p < 0.001$ $F_{4,112} = 19.43$	$p < 0.001$ $F_{4,112} = 8.41$
r^2		0.84	0.72	0.90	0.65	0.55
p value		$p = 0.03$	$p = 0.07$	$p = 0.02$	$p = 0.10$	$p = 0.15$

pipetting worm solutions under their elytra and on their mouth and anus. Two days later, inoculated females were paired with an unrelated worm-free male and allowed to mate overnight in small plastic containers. The next morning, males were removed and the mated females were provided with a freshly thawed mouse carcass and allowed to rear their broods until the point of larval dispersal. We tightly controlled carcass mass to ensure uniformity across our treatment groups given the known association between carcass mass and beetle reproductive fitness. Carcass mass ranged from 20 - 24.11g with an overall mean (± SE) of 22.31 ± 0.9 g. There were no significant differences in carcass mass between our treatment groups (ANOVA: $F_{4,113} = 0.169$, $p = 0.954$). When the beetle larvae dispersed from the carcass, we measured brood size, total brood mass and mean larval mass for each brood, as well as the number and fraction of eclosing adults.

To quantify nematode transmission between *Nicrophorus* individuals,

we measured the number of worms transmitted between experimentally inoculated beetles and worm free recipients. Transmission was examined between mating adults and from mothers to offspring.

Nematode transmission was quantified bidirectionally between males and females (i.e. female donors to male recipients and male donors to female recipients). Individual adults were first inoculated with different worm densities, as outlined above, and then maintained for 2 days in small boxes containing 1-2 cm of sterile soil. Next, these individuals were transferred to a new box with sterile soil, and paired with a nematode-free individual of the opposite sex for mating. Two days later, both individuals were sampled to determine nematode densities.

To estimate worm transmission from parental females to offspring, females were inoculated with different worm densities, allowed to mate with a worm-free male, and then provided a fresh carcass for breeding. When beetle offspring eclosed, they were sampled to estimate nematode densities.

Statistical analyses

There were 17/20 successful broods in the 'no nematodes' treatment, 26/32 in the '10 nematodes' treatment, 25/32 in the '100 nematodes' treatment, 26/32 in the '1000 nematodes' treatment, and 19/32 in the '10000 nematodes' treatment. A Shapiro-Wilk test based on successful broods was used to test for normality in experiments examining the effects of nematode infection on larval fitness (All < 0.05). We used ANCOVA to test for significant effects on a) brood size, b) total brood mass, 3) mean larval mass, 4) number of eclosed adults and 5) fraction of eclosed larvae, while controlling for carcass size. The relationship between brood size and mean larval mass within each nematode treatment was examined using linear regression. We used a generalized linear model (GLM) to test for interactions between nematode infection and the trade-off between brood size and mean larval mass. Differences in nematode transmission were estimated using t-tests. All analyses were performed using SPSS version 24 (IBM SPSS Inc., Chicago, IL, U.S.A.).

Results

Nematode identification and infection densities in wild beetles

The partial nematode 18S rRNA gene sequence (~ 900bp) was BLASTed against the NCBI database and showed 95% identity to *Rhabditoides regina* strain DF5012 (AF082997) and was clearly distinct from *Rhabditis stammeri*, the nematode species already described from *N. vespilloides*. With the resolution we have from this sequence, it remains uncertain if our isolate is truly *R. regina* or an as yet undescribed species; further sequencing will be required in a later study to more fully resolve its taxonomy. For ease of presentation we hereafter tentatively refer to our isolate as *R. regina*. Although *R. regina* has not been previously reported in *Nicrophorus*, it has been reported as a parasite in scarabaeid beetle larvae (38).

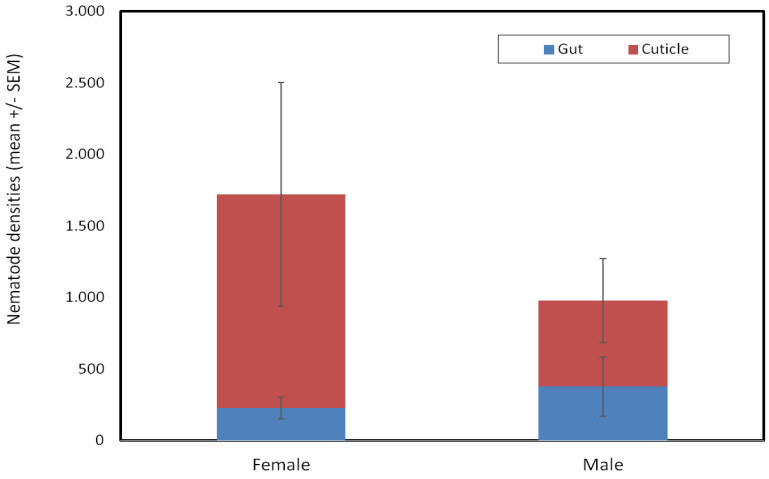


Figure 1. Nematode densities on field-caught ***N. vespilloides***.

Nematode densities were quantified from field-caught beetles, and we observed no overall differences in densities between males and females or between the gut and cuticle samples (all tests NS) (Figure 1). The mean number of nematodes in females was (Mean ± SE: 1,720.47 ± 828.45) and for males was (Mean ± SE: 978.27 ± 372.75). The number of nematodes in females and males was highly variable (females: 40 - 12,101; males: 10 - 4,608 worms).

Effect of different starting nematode densities on larval fitness

Nematode infections are highly costly to beetles. We observed significant treatment effects associated with different nematode densities on all fitness parameters after controlling for carcass mass (Table 1). In addition, we observed a significant negative linear relationship between the number of inoculated nematodes and mean brood size (r^2 = 0.84, p = 0.03) and mean larval mass (r^2 = 0.9, p = 0.02) (Figure 2). Brood size declined nearly 3-fold in broods with the highest nematode densities (Mean ± SE: 11.21 +/- 2.15 larvae/brood) compared to nematode-free broods (Mean ± SE: 27.25 +/- 3.03 larvae/brood), while mean larval mass declined roughly 15% from 0.181 +/- 0.006 g in broods without nematode infections to 0.156 +/- 0.007 g in broods where females were inoculated with 10,000 worms.

In addition to these direct negative effects of nematode infection, we observed a significant interaction between worm density and the trade-off between brood size and average larval mass (F = 9.332, df = 1, p = 0.003, Figure 3). Most interestingly, whereas there was a significant trade-off between brood size and average larval mass in worm-free beetles (r^2 = 0.50, p = 0.001), there was no association in broods produced by females infected with worms at any of the treatment densities (10 nematodes: r^2 = 0.04, p = 0.321; 100 nematodes: r^2 = 0.001, p = 0.896; 1,000 nematodes: r^2 = 0.072, p = 0.185 and 10,000 nematodes: r^2 = 0.0004, p = 0.935).

Transmission between sexes and from mothers to offspring

We inoculated male or female beetles (donors) with different nematode Nematode infections are highly costly to beetles. We observed significant treatment effects associated with different nematode densities and then measured worm transmission to opposite sex recipients during mating. As shown in Figures 4A and 4B, we observed intersexual transmission of nematodes in both directions, although this varied with worm density and the sex of the donor. For both donor sexes, when the initial density of nematodes was 10, there was neither transfer nor retention of worms. Transmission occurred at all other initial worm densities.

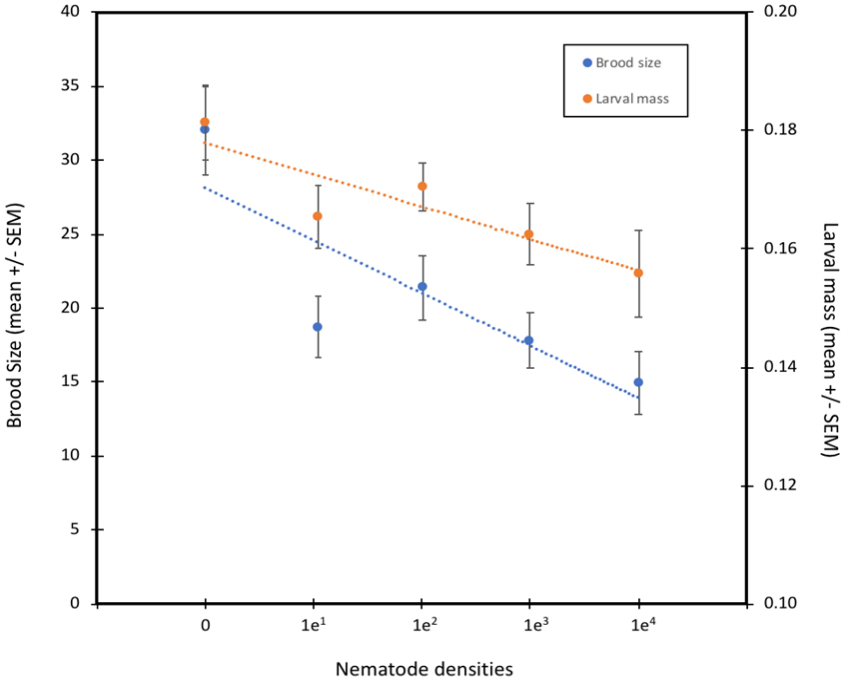


Figure 2. Decline in brood size and larval mass as a function of initial nematode numbers on mated females.

At inoculation densities of 100, worm numbers declined slightly in both male and female donors; however, transmission occurred effectively and there were no significant differences in the final worm densities of donors or recipients (t_5 = -2.05, p = 0.095). Worm densities were retained at initial values when donor females were inoculated with 1,000 worms but were significantly reduced when the initial inoculum was 10,000 worms (t_5 = -23.2, p < 0.001). Final worm densities in males and females (recipients and donors, respectively) did not differ at either inoculum density. In addition, there were no differences in final worm densities between beetles inoculated with 1,000 or 10,000 worms, suggesting an estimated carrying capacity of roughly 2,000 worms per adult beetle (mean ± SE: 1770 +/- 238.3).

For male donors, both transmission and retention were reduced compared to female donors. At initial densities of 1,000 or 10,000, we observed significant reductions in donor and recipient worm densities overall (1,000: t_5 = -56.64, p < 0.001; 10,000: t_5 = -83.5, p < 0.001). There were no differences in final worm densities at all inoculum sizes > 10, suggesting a carrying capacity of

approximately 125 worms/adult beetle (mean ± SE: 123 +/- 41.6). Although both sexes can transfer nematodes to the opposite sex during mating, female transfer and retention is approximately 10X higher in females than males (t_{34} = 6.3, $p < 0.001$).

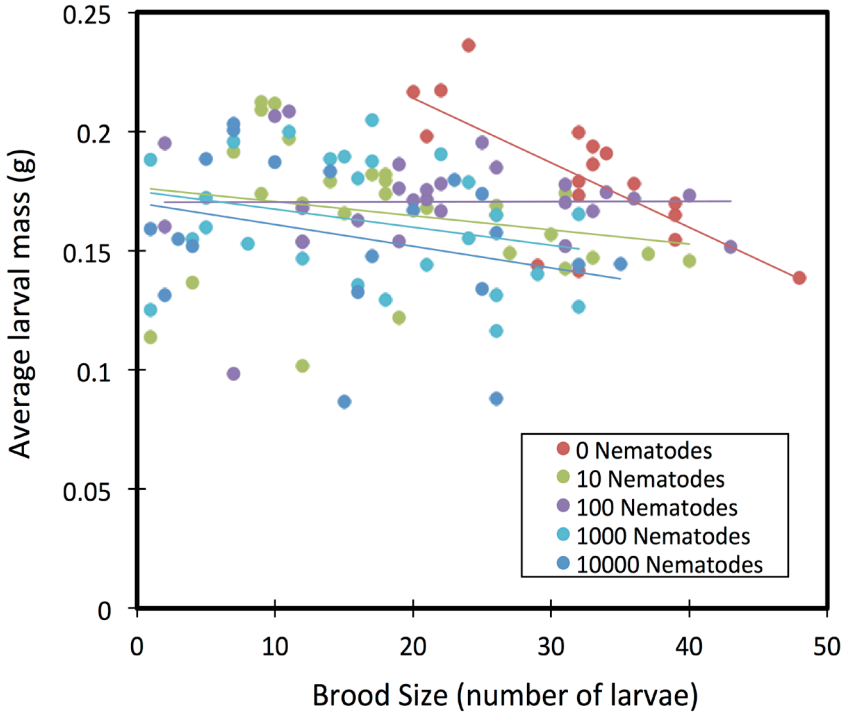
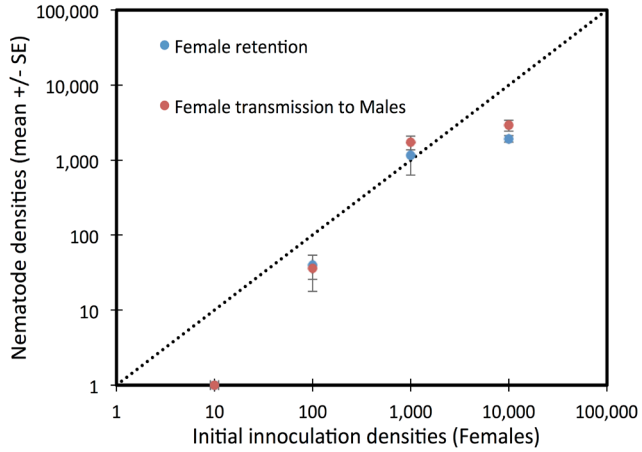


Figure 3. The relationship between average larval mass (g) and total brood size. Lines represent linear regressions. All are NS except for the nematode-free treatment.

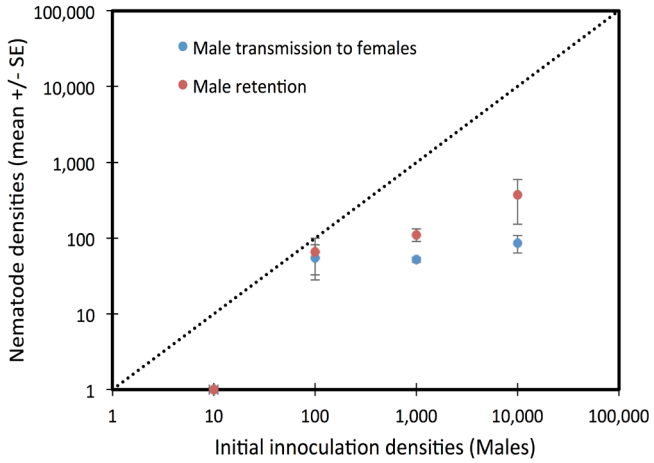
Next, to measure transmission from mothers to offspring, we inoculated mated females with different densities of worms and then allowed them to rear broods, after which we quantified the number of nematodes on eclosing pupae (Figure 4C). For all inoculum sizes other than 10,000, we observed significant transmission from mothers to larvae (one-sample t-test: 10: t_6= 2.78, p = 0.039; 100: t_{10} = 2.43, p = .036; 1,000: t_{13} = 3.02, p = 0.01). In addition, worm densities on eclosing larvae were significantly or marginally greater than maternal inoculum densities (one-sample t-test: 10: t_6= 2.6, p = 0.047; 100: t_{10} = 2.0, p = .072; 1,000: t_{13} = -2.394, p = 0.032). Finally, we observed no significant

differences in the final densities of worms on eclosing larvae from the 10, 100, and 1,000 treatments (one-way ANOVA: $F_{2,30} = 0.79$, $p = 0.463$), with a mean of approximately 500 nematodes per eclosed individual (mean +/- SE: 494.1 +/- 120.2). Unexpectedly, we found negligible transmission when mothers were initially inoculated with 10,000 nematodes, a likely artefact attributed to the extremely high rate of larval mortality in this treatment group (brood success ~ 7%).

(A)



(B)



(C)

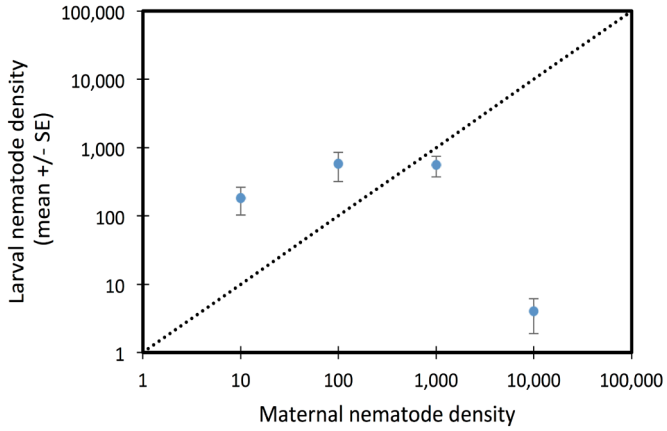


Figure 4. Nematode transmission between mated pairs and between generations. (A) transmission from females to males; (B) transmission from males to females; (C) transmission from mothers to offspring.

Discussion

Because phoretic species have limited dispersal capacity on their own, they rely on the greater motility of other species to coordinate their longer-distance transport across the environment. Such transport is beneficial and typically obligatory for the phoront, leading to the belief that phoretic species should not harm their hosts, or risk compromising their transmission. However, this expectation is not always realized, and the effects of apparently phoretic species can range from mutualism to parasitism. For example, female bark beetles that carry mites produced larger and heavier offspring, suggesting phoront-specific benefits (39). By contrast, mites carried by the red palm weevil significantly reduce beetle longevity, indicating severe costs (40). The factors that determine these different outcomes are varied and context-dependent, and show likely parallels to the diverse factors that influence the evolution of parasite virulence. As with parasites, the virulence of phoretic species may increase if this correlates with increased transmission. It could also increase in cases where the phoront interacts with multiple host species, thereby reducing reliance on any single host, or where there is competition between different genotypes of a single phoront species. Phoront virulence may also vary across different stages of host development, depending on the coupling between the

developmental/dispersal stage of the phoretic species and that of its host. To establish the baseline against which to examine these issues, our aim here was to quantify the fitness effects and transmission between adults and across generations of phoretic nematodes of *N. vespilloides*.

Our results provide the first evidence for an association between *Nicrophorus* beetles and the nematode *R. regina* or a novel species closely related to *R. regina*, a pathogenic species only previously known from the haemocoel of scarab beetles (41). Because it causes high mortality in scarabs and releases bacteria during infection, *R. regina* has been characterized as an entomopathogen that feeds on the bacteria that proliferate within the beetle cadaver (42, 43). Here, although we find that *R. regina* harms *Nicrophorus*, its behavior and transmission is more consistent with phoresy. In particular, we observed massive population growth of nematodes on the carcass itself (possibly due to consumption of bacteria on the carcass) and also conspicuous worm nictation upon disturbance. Nictation is a behavior commonly seen in phoretic nematodes that is thought to facilitate dispersal. It involves standing upright and waving in all directions, thereby attracting potential hosts (44). In addition, the strong sex-bias in nematode densities both in the field (Figure 1) and the laboratory (Figure 4) is consistent with the idea that worms are maximizing dispersal potential by preferentially associating with the sex most likely to colonize a breeding resource (a carcass). Similar biases have been observed in other phoretic nematodes (9, 45) and mites (46–48). Our field collections reveal that this species is maintained in high, although variable, densities in male and female wild beetles (Figure 1) while further studies in the benign conditions of the lab (YW unpublished) have shown that they are also stably maintained within laboratory populations of burying beetles at even higher densities. Although *Nicrophorus* nematodes were believed to have no or marginal effects on beetle fitness, our results indicate that this is not the case. Worm infections cause significant harm to beetles and the extent of this harm scales with worm density for both brood size and mean larval mass (Figures 2 and 3, Table 1), two central measures of adult and larval fitness, respectively. In addition, nematodes are transferred at high rates between adults and from parents to offspring (Figure 4), suggesting that despite host harm, transmission potential is maintained.

We find strong density-dependent effects of nematodes on *N. vespilloides*. However, even though we see a significant negative relationship between worm

numbers and e.g. brood size and mean larval mass, much of the maximum cost observed at the highest worm density (10,000) is already observed at the lowest inoculum size we used (10 worms). In other words, of the ~ 50% decline in brood size in beetles inoculated with 10,000 worms, around 80% of this decline is already apparent in beetles inoculated with only 10 worms. This result is consistent with the idea that worms are proliferating extensively on the carcass where they can then go on to infect larvae, which seems to occur whether the initial number of colonizing worms is high or low. This result also explains why nematode transmission from parents to offspring has no lower threshold (Figure 4C), in contrast to the threshold of ~100 worms needed for transmission between breeding adults (Figures 4A and 4B). Equally, while the densities of worms on larvae tends to exceed the inoculum density on females (consistent with proliferation), this is not the case for intersexual transmission, suggesting that nematode reproduction does not occur in this context and that worm transmission between adults suffers from stochastic loss if worms are initially rare.

Less clear are the factors that are responsible for the harm worms cause to reduce beetle fitness. Nematodes can reduce fitness in several ways. Before establishing broods, infected females may either forgo egg laying or reduce the number of eggs they lay to reduce the costs of rearing a brood while interacting with nematodes. Although we don't see any influence of nematode inoculation density on the failure to lay, we did not estimate egg numbers directly so are unable to assess the effects of nematodes on female reproductive investment. This will undoubtedly be of interest in future studies. Nematodes could potentially cause indirect harm to beetle larvae by competing with them for space or resources, or possibly, by physically interfering with larvae while they consume the carcass. Phoretic nematodes are bacterivores, so direct resource competition with beetle larvae seems unlikely, unless some part of beetle nutrition is also microbial (directly or indirectly) (49). Competition for physical space may occur if nematode densities are sufficiently high to prevent larval feeding or access to parts of the carcass. This type of interference could also explain the absence of a trade-off between brood size and larval mass (Figure 3), since much of this effect is driven by the reduction of larval size in smaller broods (< ~10 larvae/brood). Direct harm could possibly arise at different stages of development. Eggs could be pierced, something observed by *Nicrophorus* phoretic mites, *Poecilochirus carabi* (50), or otherwise damaged by nematodes. Larvae could also be directly harmed by worms during their growth (51). It is notable that worms are not only transported

on the surface of beetles, but are also recovered from within the digestive and reproductive tracts (Figure 1), indicating an ability to invade host tissue (52, 53). Internalized worms may obtain nutrients from the larvae or otherwise hinder their growth and development (52). At present, this remains unknown because we lack intermediate samples of larvae themselves, and instead have focused on characterizing the transmission route of worms from mature adults through to newly eclosing adults. It will be interesting in future work to sample worm densities in developing larvae to better understand how and when worms inflict their damage. In addition, it will be worthwhile to determine the extent of transmission via dispersing adults on the same carcass. This will be especially important for males who disperse from the carcass before larvae are fully matured, as this second source of nematode transmission may serve to reduce the density of worms on individual larvae.

Although our results make clear that *R. regina* is common in field-caught beetles and can persist through a complete beetle life-cycle, there are important limitations to our study. Most notably, our fitness experiments were carried out in the lab in the absence of other species that could either mitigate or exacerbate the harm caused by nematodes. *Nicrophorus* beetles carry other phoretic species: many different species of mites (32), possibly other species of nematodes (41, 54) and potentially diverse genotypes of individual species that compete with one another for transmission, thereby affecting virulence (55). While there is no evidence of simultaneous carriage of different nematode species, this has been observed in other insect:nematode associations and is possible here, too (41). On the other hand, mites and nematodes always co-occur in *Nicrophorus*. Wilson and Knollenberg (1987) found that the effects of mites varied from harmful at high densities to neutral or even beneficial at lower densities. They also found that mites reduce the burden of nematodes on eclosing adults by up to 6-fold, from ~ 18,000/individual to ~ 3,000/individual (31). This reduction could have different causes, from direct consumption of nematodes to other types of interference competition; regardless, their experiments make clear the importance of examining the effects of phoretic species in the context of the entire community. This includes mites and nematodes, but also should include the microbial species that live on and within the beetles and carcass, and also the microbes that are carried within the nematodes, especially because these bacteria may be directly associated with nematode entomopathogenicity (51, 56).

Nematodes have a broad continuum of effects on their host species (57), and transitions between levels of harm appear to be widespread and context-

dependent even among closely related hosts (58). Although phoresy is often assumed to be a commensal interaction that benefits worms without harming their hosts, the results of our study suggest that this assumption is incorrect—at least in our model system. We show that the phoretic worms of *N. vespilloides* are detrimental to beetles under laboratory conditions even though they rely on beetles for transmission to new resources. However, this harm may be inevitable and unavoidable if worm transmission requires proliferation. It will be important to determine if this is similarly true for other phoretic associates of animals. It will also be important to examine interactions between nematodes and *Nicrophorus* in the field, where associations with other phoretic species may modify the effects of nematodes on their hosts.

Authors' contributions

YW and DR conceived the ideas and designed methodology; YW collected the data; YW and DR analysed the data and co-wrote the manuscript. Both authors contributed critically to the drafts and gave final approval for publication.

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Data Accessibility

All data in this paper will be deposited with Dryad upon acceptance and are also available directly from the senior author.

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Chapter 6

Summary, discussion and perspective

Summary

Insects gut microbiomes influence host health and development, with effects varying from harmful to beneficial in different systems and contexts (1, 2). In order for insects to reliably maintain the beneficial associations they have with their symbionts, insects have evolved diverse methods to acquire their gut mutualists, from strict vertical transmission from parents to offspring, to horizontal transmission that relies on a form of environmental transfer (3, 4). In addition to mutualists, other microbes can also invade insect gut systems that can be pathogenic, or produce toxins that may harm their hosts (5–7). Insects have evolved a multiplicity of behavioural and antimicrobial strategies to compete against these microbial competitors, such as the expression of insect immunity derived antimicrobial peptides (AMPs) (8–10). In addition, insect symbionts can also help their hosts exclude invading microbes via a process called colonization resistance (11–13). This type of antimicrobial resistance takes place within the host gut community, and benefits both the hosts and their gut symbionts (12, 14). However, it is still unknown if and how insect hosts influence this type of colonization resistance, and whether it is caused by the specific composition of host microbiomes. In this thesis, by studying the burying beetle *Nicrophorus vespilloides*, I was able to test the associations between insect ecology and its microbiota, along with the effects of the competitive interactions within the host gut community on host ecology. My study will help us to better understand the complex interactions between insects and their microbiota.

The burying beetle *N. vespilloides* (Coleoptera, Silphidae) is a holometabolous insect, which undergoes a complete metamorphosis. These insects are reared on decomposing carcasses where they encounter dense and diverse bacterial populations (15). The carrion-borne microbes that are encountered by beetle larvae can invade the beetle gut and reduce beetle fitness throughout their development (16). Notably, parental beetles provide different types of parental care (pre-hatch and post-hatch care)

to the offspring that can reduce the microbial challenges experienced by larvae. These behaviours include, among others, the modification of the carcass by secreting antimicrobials and changes to larval nutrition via direct regurgitation from parents to offspring (15, 17). In this thesis, I tested if the microbial interactions within the *N.vespilloides* gut microbiota could also contribute to the antimicrobial strategies of beetles. I further clarified how parental care facilitates the transmission of gut microbiota to larvae during their development in the presence of high bacterial densities on the carcass. In addition to microbial interactions, *N.vespilloides* is also associated with mites and nematodes. By examining interactions with nematodes, in this thesis I was able to broadly examine a multiplicity of interspecific interactions during insect development and to assess their effects on *Nicrophorus* ecology.

N.vespilloides fitness is challenged by environmental microbes during the egg stage

In **Chapter 2**, I assessed how microbial challenge influences the survival of *N.vespilloides* eggs by varying the levels of environmental exposure to bacteria. I show that egg survival is reduced following exposure to the carrion environment. The negative effect from environmental microbes can be offset by sterilizing the eggs and can be reintroduced by exposure to environmental bacteria. I further studied if *Nicrophorus* eggs express any intrinsic immunity. First, I determined that *Nicrophorus* eggs possess the immunologically active serosa. I next quantified the immune response mediated by the serosa after bacterial injections. Surprisingly, and in contrast to other insects (18), I found that the serosa doesn't appear to regulate AMP production, which indicates an absence of immune response in eggs. In addition, *Nicrophorus* eggs show limited defence against desiccation—another serosa-dependant trait. I finally consider these results in an evolutionary context. Similar to *Drosophila melanogaster* (19), *N.vespilloides* eggs seems to trade-off rapid development for an explicit immune response.

Parental care facilitates the gut microbiota transmission of *N.vespilloides*

In **Chapter 3**, I manipulated the types of *N.vespilloides* parental care to offspring larvae, and I monitored the dynamics of gut microbiome colonization in terms of bacterial density and composition through development. By using a combination of MALDI-TOF Biotyping and 16s rDNA sequencing,

I show that the larval gut microbiome undergoes similar dynamics, in terms of density, regardless of the duration of parental care; however, bacterial composition is strongly determined by parental care. I also discovered that there is an aposymbiotic stage during larval pupation (where pupae become bacterially sterile), after which pupae are recolonized at eclosion with bacteria similar to those found on the molted larval cuticle and on the wall of the pupal chamber. I determined that pre-hatch care facilitates and ensures the colonization and transmission of *N.vespilloides* gut microbiota from parents to offspring. Additionally, I find that environmental bacteria predominantly colonize the larval gut when parental care is completely absent. These results together suggest that competitive interactions within the *N.vespilloides* gut community might be a factor in the colonization and persistence of *N.vespilloides* gut microbiota.

Colonization resistance functions in the persistence of gut bacteria and provides resistance against pathogens

In **Chapter 3**, we concluded that the endogenous microbiota outcompete the carrion-associated bacteria for colonization of the larval gut niche. In **Chapter 4**, I was able to test this hypothesis directly by using competition assays in vivo. I set up experiments with four bacterial species, of which *Providencia rettgeri* and *Morganella morganii* are abundant species in the *Nicrophorus* gut, and conversely *E.coli* and the pathogen *Serratia marcescens* (20) are commonly found in the environment. I first inoculated these four strains alone into *N.vespilloides* larvae, and the results show that the bacterial species vary in their colonization capability within the beetle gut. I next co-inoculated larvae with different bacterial combinations simultaneously or in series. I show that endogenous species significantly outcompete foreign species within the *Nicrophorus* gut, regardless of the inoculation order. Therefore I confirm that *N.vespilloides* gut bacteria provides colonization resistance against the pathogen *S. marcescens*. To further determine and illuminate the potential benefit of this colonization resistance, I quantified the fitness effects of native gut flora in developing larvae. Results show that the gut microbiota benefit *N.vespilloides* by increasing both parental and larval fitness. I discuss these results in both ecological and evolutionary perspectives, and suggest that parental behaviour and microbial competition interact to influence the transmission and colonization of *Nicrophorus* gut microbiota.

Influence of phoretic nematodes on *N.vespilloides* fitness

The last experimental part of my thesis (**Chapter 5**) focused on the interspecific interactions between *N.vespilloides* and nematodes. Using direct microscopy counts, I first quantified the number of nematodes in both wild and lab beetle samples. I find no significant difference of nematodes in densities between males and females. Next, I determined that the phoretic nematode species of *N.vespilloides* is *Rhabditoides regina*. I characterized the efficacy of nematode transmission across partners and generations. My results show that nematode transmission occurs within the *N.vespilloides* breeding context between mating partners and also from parents to offspring. Notably, nematode transmission from parents to offspring can start with an extremely low inoculation of ~ 10 worms. In contrast, nematode transmission across mating adults shows a threshold of ~ 100 worms. Finally, I estimated the effect of different starting nematode densities on the larval fitness, and I showed that the negative effects of nematodes on *N.vespilloides* larvae are seen even with a very low inoculum sizes.

Discussion and perspective

In this thesis, I have illuminated the evolution and ecology of gut symbionts of the social insect, *N. vespilloides*, and examined some of their benefits to their host. I linked the extensive parental care of *Nicrophorus* beetles with their gut microbiota colonization ecology.

Nicrophorus beetles are reared on highly decaying carcasses, which exposes larvae to severe microbial challenge that begin at the egg stage (**Chapter 2**) (17). However, neither direct antimicrobial provisioning from parents nor intrinsic immunity can be found in *Nicrophorus* eggs to cope with these microbial threats. This is similar to other insects such as *D. melanogaster*, which feeds on rotting fruits. From an evolutionary perspective, our results suggest that *Nicrophorus* eggs might be selected for rapid growth and this results in a trade-off between developmental speed and immune competence (21, 22). Still, these results need to be interpreted carefully, because *Nicrophorus* eggs may be not be as unprotected as we believe. Many potential forms of protection, such as the antiseptic volatiles from parents or the effects of bacterially produced antimicrobial regents in soil have yet not been tested (23–25), and this will require a more complex experimental set-up in future

research. This work will extend our knowledge about the animal parental strategies on maintaining the offspring fitness and developments.

Although parents do not apparently protect *Nicrophorus* eggs, parental beetles provide a comprehensive set of caring behaviours for their hatched larvae, including carcass preparation (pre-hatch care) and direct larval feeding (post-hatch care). Throughout both stages of parental care, parent beetles transmit their endogenous gut microbiota to offspring larvae (Chapter 3), and we showed that these native microbes can increase larval fitness under pathogen challenge (**Chapter 4**). Interestingly, with pre-hatch care alone, offspring larvae can also acquire and assemble many of the prominent members of their endogenous gut community. We found this was not due to the inability of environmental bacteria to colonize larvae, because larvae reared without parental care are still colonized by the carcass-borne bacteria at high densities. Also, larvae receiving pre-hatch care alone are partially colonized by the environmental bacteria (**Chapter 3**). We thus suggest that endogenous bacteria outcompete foreign species within the larval gut, which is an essential factor in stable microbiota transmission. The route by which parents manipulate the carcass during pre-hatch care to bacterial facilitate transmission to larvae are only partly understood. Parents open the carcass abdomen and coat the carcass with lysozyme-like secretions. These behaviours may bias carcass microbiota towards aerobic and Gram-negative species, respectively (17, 25, 26), and further influence gut microbiota establishment. Previous research has shown that *Nicrophorus* suffer a dramatic fitness cost when reared on an aged carcass (16). In future work, it would be interesting to more closely examine the differences between fresh and aged carcass microbiomes after parental preparation, and carry out further work on larval microbiota transmission from aged carcasses, where parental care is dramatically challenged. In **Chapter 3**, I also show a clear aposymbiotic stage during beetle pupation, which is consistent with several fly and mosquito species (27, 28). Interestingly, following this aposymbiotic stage, eclosed beetles recover their gut bacteria in density and the composition of this microbiota significantly overlap with those present prior to pupation. This indicates a reliable mode of parent-offspring transmission of *Nicrophorus* gut microbiota, and further suggested that there may be beneficial functions of this microbiome that potentially limit larval infections derived from the carcass environment. I address this question in this thesis in the laboratory, and in the future it will be exciting to extend our research to field derived beetles that are exposed to more complex bacterial communities. This part

of work will help us to better understand the mechanisms and associations of maternally transmitted symbionts in other insects e.g. Aphid or mammals.

In **Chapter 4**, I examined the hypotheses proposed in **Chapter 3**. I demonstrate that native bacteria outcompete foreign species within the larval gut, which could play an important role in the stability of *Nicrophorus* gut microbiota (29). I also show that even after 24 hours of head-start colonization by *S. marcescens*, *P. rettgeri* can still successfully invade the larval gut and replace *Serratia*. This outcome differs from the priority effects observed in other models (30), although why this is the case is not fully clear. One possibility is that endogenous species are better colonizers of the larval gut than non-native species, a result confirmed in **Chapter 4**. This implies that bacterial colonization within larval gut is highly specific. However, we still have little understanding about what factors between bacteria, their host and the interactions between these factors drive this specificity in *N.vespilloides*. One of the factors could involve specific co-evolved genes between *Nicrophorus* and certain bacterial species that favour colonization. For example, the cell adherence gene ccf in Bacteroides fragilis is essential for the association with colonic mucus, and thus plays an important role in Bacteroides colonization in the mice gut. The mice colonic crypts meanwhile represents a stable niche even after microbial disruption by other bacteria or antibiotic treatments (31). In addition, around 15% of protein-coding genes in *Snodgrassella alvi* are found to be essential for gut colonization in honeybees, and some factors like cell O-antigens and type IV pili (T4P) function in attachment on the hindgut epithelium. Another factor could be derived from host metabolic and biosynthesis pathways, which promote and favour the specific colonization of bacteria. This has been also found in the specific colonization of S. alvi within the honey bee gut (32). Genome-wide screening and transcriptome targeting could address these questions in our system, for example, non-endogenous strains with transformed genes of specific colonization factors might offset prior competitive advantages in the endogenous species. Other factors such as host triggered direct bacterial killing (33), microbiota-mediated host immune responses (34) and the physical structure of host intestinal tract (35) might influence the process of colonization and colonization resistance. Further, the specificity mediated bacterial colonization resistance will shed light on the host-symbionts cooperated therapeutics in the future medical research.

In **Chapter 5,** I provide evidence of an association between the phoretic

nematode *R. regina* and *N.vespilloides*, I further show that transmission of this nematode relies on *N.vespilloides* social behaviour and thus persists through larval development. I also show a strong negative effect of *R. regina* on *N.vespilloides* fitness.

The reason for the negative effects of *R. regina* to beetles could be diverse: 1) nematodes that reach high densities might directly compete with beetle larvae for food resources; 2) because nematodes feed on bacteria and potentially associate with pathogens, this could convert nematodes into incidental vectors of pathogens (36). Further research will be required to determine the mechanisms underlying the negative effects of *R. regina* on *Nicrophorus* beetles.

In nature, other phoretic mites and nematodes might simultaneously interact with *Nicrophorus* beetles. Due to the potential of mite predation on nematodes (37) and the density-dependent effects of mites on *Nicrophorus* fitness (38), there might be an ecological balance in terms of the number of associated species within the *Nicrophorus* living context. All these would be interesting to further examine in the future.

Conclusions

In conclusion, I demonstrate vertical transmission of gut microbiota by parental *N.vespilloides* that persists across larval development. I next demonstrate the apparent mutualism between *N.vespilloides* and their gut symbionts. And interestingly, I show that colonization resistance plays an important role in the transmission and colonization of *N.vespilloides* gut microbiota. Finally, I report a new species of phoretic nematode, *R. regina* that associates with *N.vespilloides* and which is significantly reduces beetle fitness. My data contributes to an increased understand of the relationships between insect parental care and the social transmission of beneficial gut bacteria. Future studies will shed light on the mechanisms that regulate host-symbiont mutualism in this model system.

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Chapter 7

Nederlandse Samenvatting – Summary in Dutch

Darmmicroben in insecten beïnvloeden de gezondheid en ontwikkeling van de gastheer, de effecten van de darmmicroben kunnen zowel positief als negatief zijn in verschillende systemen (1, 2). Om mutualistische darmmicroben aan te trekken hebben insecten diverse methodes geevolueerd. De methodes om de darmmicroben aan te trekken verschillen per insect, sommige zijn sterk afhankelijk van verticale transmissie of van horizontale transmissie (3, 4). Naast mutualistische microben zijn er ook invasieve soorten bekend die het darmsysteem van een insect aanvallen door middel van giftige stoffen en uiteindelijk kunnen leiden tot het overlijden van de gastheer (5–7). Om zich tegen deze pathogenen te verweren hebben insecten verschillende strategieën ontwikkeld in bijvoorbeeld gedrag of expressie van antimicrobiële peptiden (AMPs) (8–10). De insect symbionten kunnen ook de gastheer helpen door andere microben buiten te houden, bijvoorbeeld door het verhinderen van kolonisatie van pathogenen genaamd kolonisatieresistentie (11–13). Deze manier van afweer is positief voor zowel de gastheer als de microbe. (12, 14). Echter is het nog steeds onbekend hoe insecten de kolonisatieresistentie beïnvloeden en of de kolonisatieresistentie voortkomt uit een specifieke samenstelling van microben. Tijdens deze thesis heb ik in de doodgraver *Nicrophorus vespilloides* de associaties tussen insect ecology en zijn microben onderzocht, samen met de effecten van de competitieve interacties in de darmflora en de gevolgen op de gastheer. Mijn studie zal ons de complexe interactie tussen de gastheer en zijn microbioom beter helpen begrijpen.

De doodgraver *N. vespilloides* (Coleoptera, Silphidae) is een holometabool insect welke een volledige metamorfose ondergaat. Deze kever broed op ontbindende kadavers waar ze in aanraking komen met een groot, divers scala bacteriële populaties (15). De microben van de kadavers kunnen de darmen van de keverlarve infecteren met als gevolg een achtergestelde ontwikkeling (16). Opmerkelijk is de ouderlijke zorg die de kevers geven, zowel voor als na het uitbroeden, welke resulteert in een lagere kans op besmetting met microben. Voorbeelden van deze zorg zijn het uitscheiden van antimicrobiële stoffen die het kadaver modificeren en het veranderen van het voedsel voor de

larven door middel van regurgiteren (15, 17). In deze thesis heb ik onderzocht of de microbiële interacties in de darmen van *N. vespilloides* ook bijdragen aan de antimicrobiële strategieën. Zo heb ik kunnen verduidelijken hoe de ouderlijke zorg het overdragen van de darmmicroben faciliteert op een ontwikkelende keverlarve in bijzijn van een karkas met hoge bacterie dichtheid. Buiten de microbiële interacties wordt *N. vespilloides* ook geassocieerd met mijten en nematoden. Door de interacties met nematoden, te onderzoeken heb ik in deze thesis veel interspecifieke interacties tijdens de ontwikkeling van insecten kunnen bestuderen en zo de effecten op de *Nicrophorus* ecologie kunnen vaststellen.

Risico van omgevings microben op de fitness van *N.vespilloides* tijdens het eier stadium.

In **hoofdstuk 2** heb ik beschreven hoe microben de levensvatbaarheid van *N.vespilloides* eieren beïnvloeden door de milieublootstelling aan bacteriën te variëren. Een langere blootstelling aan aas resulteerde in een gereduceerde levensvatbaarheid. Door de eieren na blootstelling te steriliseren kan het negatieve effect op levensvatbaarheid ongedaan worden gemaakt, wederom blootstellen aan aas resulteerde opnieuw in verlaagde levensvatbaarheid. Vervolgens heb ik onderzocht of de *Nicrophorus* eieren het aangeboren immuunsysteem uiten. Eerst heb ik vastgesteld dat *Nicrophorus* eieren een immunologische actief serosa bevatten. Vervolgens is de gemedieerde immuunrespons van de serosa gekwantificeerd door het toedienen van bacteriële infecties. Verrassend en in tegenstelling tot andere insecten (18), vond ik dat de serosa de productie van AMP niet lijkt te reguleren. Deze bevinding wijst erop dat een immuunrespons afwezig is in de eieren. Bovendien vertonen *Nicrophorus* eieren een beperkte afweer tegen uitdroging, uitdroging wordt ook gereguleerd door de serosa. In een evolutionaire context lijkt de ontwikkeling van *N.vespilloides* veel op die van *Drosophila melanogaster* (19), een snelle ontwikkeling staat centraal en dit gaat ten koste van de aangeboren immuunrespons.

Ouderlijke zorg faciliteert de overdracht van darm microben in *N.vespilloides*

In **hoofdstuk 3** heb ik de ouderlijke zorg voor nakomelingen van *N.vespilloides* gemanipuleerd. Hierbij volgde ik de dynamiek van de darmmicrobioom kolonisatie kijkend naar bacteriële dichtheid en compositie

gedurende de ontwikkeling. Door MALDI-TOF Biotying en 16s rDNA-sequencing te combineren heb ik aangetoond dat het microbioom van de larvale darm een vergelijkbare dynamiek ondergaat in termen van dichtheid, ongeacht de duur van de ouderlijke zorg. De samenstelling van bacteriën wordt echter wel sterk bepaald door ouderlijke zorg. Ook ontdekte ik een stadium tijdens de verpopping waarin de larve volledig steriel worden. Na dit stadium en tijdens het ontpoppen worden de kevers gekoloniseerd door de bacteriën die aanwezig zijn op de muur van de poppenkamer. Hierdoor heb ik kunnen vaststellen dat de ouderlijke zorg voor het uitkomen van de eieren de kolonisatie en overdracht van de darm microben niet alleen faciliteren maar ook verzekerd. Bij volledige afwezigheid van ouderlijke zorg koloniseren grotendeels bacteriën uit het nabije milieu de darm van *N.vespilloides*. Deze resultaten samen suggereren een competitieve interacties binnen de *N.vespilloides* darmflora een rol kunnen spelen bij de kolonisatie en bestendigheid van de darm microben.

Kolonisatieresistentie functioneert in de bestendigheid van darmbacteriën en verstrekt resistentie tegen pathogenen.

In **hoofdstuk 3** hebben we geconcludeerd dat in de larvale darm van *N.vespilloides* het endogene microbioom het microbioom van het degraderende karkas weg concurreert. In **hoofdstuk 4** was ik in staat deze hypothese te testen door gebruik te maken van in vivo competitie analyses. Het experiment is uitgevoerd met vier bacteriesoorten. *Providencia rettgeri* en *Morganella morganii* zijn bacteriesoorten die een veel voorkomen in de darm van *Nicrophorus*. De bacteriesoorten *E.coli* en de pathogeen *Serratia marcescens* (20) komen veel voor in de leefomgeving van *Nicrophorus*. Eerst heb ik alle vier de bacteriestammen gescheiden geïnoculeerd in *N.vespilloides* larve, hierdoor kon ik aantonen dat de bacteriesoorten variëren in hun kolonisatie vermogen binnen de darm. Vervolgens heb ik, gelijktijdig aan dit experiment, larven geïnoculeerd met een combinatie of serie van verschillende bacterie. Met dit experiment heb ik aangetoond dat endogene soorten in de *Nicrophorus* darm significant beter overleven dan uitheemse soorten, ongeacht de volgorde van enten. Zo kon ik bevestigen dat de *N.vespilloides* darmbacterien resistentie bieden tegen kolonisatie van de pathogeen S. *marcescens*. Om de potentiële voordelen van deze kolonisatieresistentie verder te verhelderen en te bepalen heb ik de fitness effecten van de inheemse darmflora bij het ontwikkelen van de larven gekwantificeerd. De resultaten

tonen aan dat *N.vespilloides* profiteert van de darm microbioom door de fitness van zowel de larve als de ouder te vergroten. Deze resultaten bespreek ik in zowel een ecologische als een evolutionaire context. Hierbij suggereer ik dat ouderlijk gedrag, microbiële competitie en de interactie daartussen een invloed hebben op de transmissie en kolonisatie van de *Nicrophorus* darm microbioom.

Invloed van phoretic nematoden op *N.vespilloides* fitness

In het laatste experimentele deel van mijn thesis, **hoofdstuk 5**, leg ik de focus op de interspecifieke interacties tussen *N.vespilloides* en nematoden. Door gebruik te maken van een microscopische telling heb ik het aantal nematoden in zowel wilde als lab kever monsters kunnen kwantificeren. In deze telling heb ik geen significant verschil kunnen aantonen tussen de nematoden dichtheid tussen mannen en vrouwen kevers. Vervolgens heb ik vastgesteld dat de phoretic nematoden in *N.vespilloides* van het soort *Rhabditoides regina* zijn. Ook karakteriseerde ik de effectiviteit van de overdracht van nematoden over verschillende partners en generaties. Mijn resultaten tonen aan dat de overdracht van nematoden op *N.vespilloides* plaatsvindt tijdens het broeden, dit kan tijdens het paren zijn of van ouder op nakomeling. Het is noemenswaardig dat de overdracht van nematoden van ouder naar nakomeling kan plaatsvinden onder een extreem lage waarde van ~ 10 wormen. De overdracht van wormen van parende volwassenen heeft een drempel van ~ 100 wormen. Ten slotte schatte ik het effect van verschillende beginnende nematoden dichtheid op de larvale fitness en toonde ik aan dat de negatieve effecten van nematoden op *N.vespilloides* larve zelfs al bij een zeer lage inoculatie grootte worden waargenomen.

Conclusie

In deze thesis toon ik aan dat er verticale transmissie van de darmmicrobiota, van ouders naar nakomeling, plaatsvindt en aanhoudt tijdens de ontwikkeling in de soort *N.vespilloides*. Vervolgens demonstreer ik het klaarblijkelijk mutualisme tussen *N.vespilloides* en hun darm-symbionts. Een merkwaardige bevinding is dat kolonisatieresistentie een belangrijke rol speelt in de transmissie en kolonisatie van *N.vespilloides* darmmicrobiota. Ten slotte beschrijf ik een nieuwe phoretic nematode soort, *R. regina*, welke ik in verband breng met *N.vespilloides* en de fitness van deze kever significant verlaagt. Mijn gegevens

dragen bij aan een beter begrip van de relatie tussen de ouderlijke zorg door insecten en de sociale overdracht van nuttige darmbacteriën. Toekomstige studies zullen licht werpen op de mechanismen die het gastheer-symbiont mutualisme in dit modelsysteem reguleren.

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Curriculum vitae

Yin Wang was born on 22nd June, 1986, in Anqing, Anhui province, China. In 2005 he graduated from Anqing No.1 Middle School in Anhui. In September of the same year he started his Life & Environmental Science study, a shared program of National Natural Science Foundation of China and Huangshan College focused on the Phylogeography and Conservation Genetics. He finished his Bachelor program in 2009 with a research internship on Phylogeny and Anatomy diversity on intestinal system of snake under the supervision of Prof. dr. Song Huang. After obtaining his bachelor's degree with a major in Life Science, he continued with the master program in Central China Normal University with the same major. He finished his master project of "the interactions and functions research on the flagellate and its symbionts in lower termite Reticulitermes santonensis gut", with supervision of Prof. dr. Hong Yang in June 2012. He obtained his master degree in the same year.

In June 2012 he was granted a scholarship by the China Scholarship Council of the Chinese Ministry of Education for PhD study at Leiden University. In October, 2012 he arrived at Leiden and started his PhD study at the department of Microbial Biotechnology & Health at the Institute of Biology (IBL). During his PhD study in Leiden he finished his research and obtained four publications, these are all included in this thesis.

Scientific contributions

PUBLICATION LIST

1. Jacobs, C. G. C.*, Wang, Y.*, Vogel, H., Vilcinskas, A., van der Zee, M., & Rozen, D. E. (2014). Egg survival is reduced by grave-soil microbes in the carrion beetle, *Nicrophorus vespilloides*. BMC Evolutionary Biology, 14(1), 208–215. https://doi.org/10.1186/s12862-014-0208-x. (Co-first author)

2. Wang, Y., & Rozen, D. E. (2017). Gut Microbiota Colonization and Transmission in the Burying Beetle *Nicrophorus vespilloides* throughout Development. Applied and Environmental Microbiology, 83(9), e03250-16. https://doi.org/10.1128/AEM.03250-16

3. Wang, Y., & Rozen, D. E. (2018). Gut microbiota in the burying beetle, *Nicrophorus vespilloides*, provide colonization resistance against larval bacterial pathogens. Ecology and Evolution, 8(3), 1646-1654. https://doi.org/ DOI: 10.1002/ece3.3589.

4. Wang Y., & Rozen, D. E. (2018). Fitness effects and transmission of phoretic nematodes of the burying beetle, *Nicrophorus vespilloides*. Ecology and Evolution (In press). DOI: 10.1002/ece3.4570

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1. Attended National Environmental Biology Academic Symposium, 2009, Wuhan, China

2. Post presentation in BISMiS (conference of Bergey's International Society for Microbial Systematics), 2011, Beijing, China

3. Attended 26th Nederlanse Entomologendag, 2014, Wageningen, Netherlands

4. Attended 8th Congress of the International Symbiosis Society, July, 2015, Lisbon, Portugal

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1. KNAW NWO PhD Event : The Famelab workshop, Amsterdam, the Netherlands, 2012
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